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(54) Title: MODIFIED FORMS OF REPRODUCTIVE HORMONES (57) Abstract <p>The invention provides recombinant native and mutein forms of human reproductive hormones with characteristic glycosylation patterns which are influential in the metabolic activity of the protein. The invention also provides recombinant mutant forms of the human alpha subunit common to FSH, LH, CG, and TSH, to obtain hormones which also have unique glycosylation patterns. Also provided are recombinant materials to produce these subunits separately or together to obtain complete heterodimeric hormones of regulated glycosylation pattern and activity. Modified forms of LH and FSH beta subunits which enhance the rate of dimerization and secretion of the dimers or individual chains are also disclosed.</p>		

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MODIFIED FORMS OF REPRODUCTIVE HORMONES

10

Technical Field

15 The invention relates to the production of human reproductive hormones with altered glycosylation patterns and activities, and to improved modified forms of the beta subunit of LH. In particular, it concerns production of
20 recombinant hormones under conditions which result in efficient production and secretion and which regulate the glycosylation pattern of the protein, and to production of modified forms of LH with enhanced secretion and dimerization properties.

Background Art

25 Human reproductive function is controlled in part by a family of heterodimeric human glycoprotein hormones which have a common alpha subunit, but differ in their hormone-specific beta subunits. The family includes
30 follicle-stimulating hormone (FSH), luteinizing hormone (LH), thyrotropin or thyroid-stimulating hormone (TSH), and human chorionic gonadotropin (CG). In all cases, the alpha subunit is a 92 amino acid glycoprotein with two
35 canonical glycosylation sites at the asparagines located at positions 52 and 78. The beta subunits are also glycoproteins; in addition to the N-linked glycosylation

exhibited by the beta chains of all four hormones, human CG contains four mucin-like O-linked oligosaccharides attached to a carboxy-terminal extension unique to this hormone. The relevance of the O-linked glycosylation is not, apparently, related to the secretion and assembly of the hormone (Matzuk, M.M. et al. Proc Natl Acad Sci USA (1987) 84:6354-6358).

Genomic and cDNA clones have been prepared corresponding to the human alpha chain (Boothby, M. et al. J Biol Chem (1981) 256:5121-5127; Fiddes, J.C. et al. J Mol App Genet (1981) 1:3-18). The cDNA and/or genomic sequences of the beta subunits have also been prepared. For CG, the beta-encoding DNA is described by Fiddes, J.C. et al. Nature (1980) 286:684-687 and by Policastro, P. et al. J Biol Chem (1983) 258:11492-11499. For luteinizing hormone, such description is by Boorstein, W.R. et al. Nature (1982) 300:419-422; and for TSH by Hayashizaki, Y. et al. FEBS Lett (1985) 188:394-400 and by Whitfield, G.K. et al. in "Frontiers in Thyroidology", (1986) Medeiros-Nato, G. et al. (eds) pages 173-176, Plenum Press, NY. These DNA segments have been expressed recombinantly, and biologically active material has been produced.

The genomic sequence encoding FSH beta chain was used to construct a recombinant expression vector containing the complete beta chain coding sequence as described in PCT application WO86/04589, published 14 August 1986. In addition genomic clones for human FSH-beta have been prepared by others (Watkins, P.C. et al. DNA (1987) 6:205-212; Jameson, J.L. et al., Mol Endocrinol (1988) 2:806-815; Jameson, J.L. et al. J Clin Endocrinol Metab (1986) 64:319-327; Glaser, T. et al. Nature (1986) 321:882-887). However, it is not clear that human FSH beta has been engineered to permit recombinant production of the dimeric hormone. (The bovine beta FSH gene has also been obtained as disclosed in Maurer, R.A. et al. DNA (1986) 5:363-369; Kim, K.E. et al. DNA (1988) 7:227-333.)

The invention constructs permit significant recombinant production of this family of hormones, in particular, FSH and LH, and further sets forth a means for regulation of the glycosylation pattern and thereby
5 greater predictability in the formulation of therapeutically useful materials.

While it is now understood that the glycosylation pattern of a particular protein may have considerable relevance to its biological activity, the importance of this pattern has largely been overlooked in characterization of glycoproteins. Emphasis has been placed
10 on the amino acid sequence as if this were the sole component of the glycoprotein. The reasons for this myopia are largely historic, but this almost exclusive focus on
15 the peptide aspect is clearly in error. For example, it is well known in the case of human CG that desialylation causes the hormone to be cleared rapidly via the liver (Morell, A.G. et al. J Biol Chem (1971) 246:1461-1467). It is also known that removal of carbohydrate internal to
20 the sialic acid residues or complete deglycosylation converts human CG into an antagonist which binds more tightly to receptor but shows decreased biological activity in vitro (Channing, C.P. et al. Endocrinol (1978) 103:341-348; Kalyan, N.J. et al. J Biol Chem (1983) 258:67-74; Keutmann, H.T. et al. Biochemistry (1983) 3067-3072; Moyle, W.R. et al. J Biol Chem (1975) 250:9163-9169). Other glycoproteins, such as, for example, tissue plasminogen activator, are also known to be altered in
30 their degree of activity when the glycosylation pattern is changed. Therefore, it appears that in order to regulate the therapeutic function of the glycoprotein hormones, it may be necessary to control both the level and nature of glycosylation.

Human FSH and LH are used therapeutically to regulate various aspects of metabolism pertinent to reproduction in the human female. For example, FSH partially purified from urine is used clinically to stimulate follicular maturation in anovulatory women with anovulatory syndrome or luteal phase deficiency. Luteinizing hormone (LH) and FSH are used in combination to stimulate the development of ovarian follicles for in vitro fertilization. The role of FSH in the reproductive cycle is sufficiently well-known to permit this sort of therapeutic use, but difficulties have been encountered due, in part, to the heterogeneity and impurity of the preparation from native sources. This heterogeneity is due to variations in glycosylation pattern. Similarly, LH is used therapeutically to alleviate conditions associated with impaired functioning of the female reproductive system.

Disclosure of the Invention

The invention provides recombinantly produced human heterodimeric hormone of the group FSH, LH, TSH and CG, by improved means and by means which offer the opportunity for control of glycosylation pattern both on the alpha and beta portions of the heterodimer. Such glycosylation control can be obtained through either the prudent selection of the recombinant eucaryotic host, including mutant eucaryotic hosts, or through alteration of glycosylation sites through, for example, site directed mutagenesis at the appropriate amino acid residues. In any event, the recombinant production of these hormones obviates the complex mixture of glycosylation patterns obtained when the hormone is isolated from native sources.

The pM²/alpha vector disclosed herein containing the minigene is particularly efficient in providing for expression of the dimeric hormones of this family. The minigene could, of course, be ligated into a variety of

host vectors and placed under the control of suitable promoters compatible with a eucaryotic host. Similarly, the beta subunit may also be provided with alternate control systems. Efficient production of these hormones using the alpha minigene is one aspect of the invention.

In another aspect, the invention is directed to specific mutants of this family with altered glycosylation patterns at the two glycosylation sites in the alpha subunit, or to alpha subunit variants containing alterations at the carboxy terminus which affect activity. Thus, in this aspect, the invention is directed to expression systems for muteins of the alpha subunit which lack glycosylation sites at the asparagine at position 52 or position 78 or both, or contain modifications at positions 88-92, and to recombinant host cells transfected with these expression systems. The modifications at positions 88-92 are especially important as they result in a diminution or elimination of the steroidogenetic activity of the hormones, but do not effect receptor binding. Therefore, analogs to the hormones produced using these modifications can be used as antagonists. The cells may be transfected with this subunit expression system singly or in combination with an expression system for a suitable beta subunit. The invention is directed also to the mutant glycoproteins with altered glycosylation or activity patterns produced by these cells.

In another aspect, the invention is directed to modified forms of LH beta chain wherein the 7-amino acid hydrophobic sequence at positions 115-121 is deleted or replaced by a hydrophilic sequence, and at least one residue selected from the group consisting of trp⁸, ile¹⁵, and met⁴² is replaced by a hydrophilic amino acid, or thr⁵⁸ is replaced by asn. In preferred embodiments, the hydrophilic amino acid most like the hydrophobic residue replaced is used as the substitute, or the amino acid, in the corresponding position in the CG beta claim is

substituted. Also preferred are embodiments wherein the hydrophobic sequence at positions 115-121 is replaced by the carboxy terminal peptide of human CG beta subunit or a variant thereof as described below. Also included within
5 the invention are DNA sequences encoding this modified LH beta and recombinant materials and methods for the production of this subunit, either alone or in the presence of alpha subunit.

In still another aspect, the invention is
10 directed to the recombinant production of FSH wherein the resulting dimer contains an extended form of the FSH beta chain i.e. the FSH beta sequence fused to the carboxy terminal portion (CTP) of the CG sequence or a variant thereof. This modified form of FSH is very efficiently
15 produced and secreted.

The carboxy terminal portion of the CG beta subunit or a variant of this carboxy terminal peptide has significant effects on the clearance of CG, FSH, and LH. This extension at the carboxy terminus has similar effects
20 on the clearance of hormones and of protein-based pharmaceuticals in general. Therefore, another aspect of the invention is directed to modified forms of peptide or protein pharmaceuticals wherein the modification comprises ligation of the CTP or its variant to the carboxy terminus
25 of said protein or peptide. Especially preferred proteins or peptides are the peptide hormones.

In other aspects, the invention is directed to pharmaceutical compositions containing the variants set forth above, and to methods to regulate reproductive
30 metabolism in subjects by administration of these glycoproteins or their pharmaceutical compositions.

Brief Description of the Drawings

Figure 1 shows a restriction enzyme map of the human FSH beta gene.

5 Figure 2 shows the nucleotide sequence of the human FSH beta gene.

Figure 3 shows the construction of the human alpha subunit minigene, and vectors for its expression.

Figure 4 shows the construction of the extended form of FSH beta subunit.

10 Figure 5 shows a comparison of the amino acid sequences of human CG and human LH beta subunits.

Figure 6 shows the construction of various LH chimeras and muteins.

15 Figure 7 shows 35-S cysteine labeled FSH or FSH beta immunoprecipitated from cell lysates and media.

Figure 8 shows the bioassay of recombinant human FSH.

Figure 9 shows chromatofocusing of recombinant and pituitary human FSH.

20

Modes of Carrying Out the Invention

Definitions

25 As used herein, human alpha subunit, and human FSH, LH, TSH, and CG beta subunits as well as the heterodimeric forms have in general their conventional definitions and refer to the proteins having the amino acid sequences known in the art per se, or allelic variants thereof, deliberately constructed muteins thereof
30 maintaining the activity of the native protein regardless of the glycosylation pattern exhibited, or mutant forms thereof having at least 90% homology with the native forms.

35

"Native" forms of these peptides are those which have the amino acid sequences isolated from human tissue, and have these known sequences per se, or their allelic variants.

5 "Mutein" forms of these proteins are those which have deliberate alterations in amino acid sequence produced by, for example, site-specific mutagenesis or by other recombinant manipulations, or which are prepared synthetically. These alterations result in amino acid
10 sequences wherein the biological activity of the subunit is retained and/or wherein the subunit has at least 90% homology with the native form.

A preferred mutein of the alpha subunit for use in antagonists of the various heterodimers has alterations
15 in the amino acids of positions 88-92.

A particularly preferred mutein of FSH beta, for example, is an "extended" FSH beta wherein the amino acid sequence comprising the carboxy terminal peptide (CTP) of hCG is fused to the carboxy terminus of FSH beta. As used
20 herein, "CTP" refers to the "extra" sequence at the C-terminus of the CG beta peptide as compared to the other related hormones. The length of the effective CTP as compared to the other beta subunits may vary slightly but it extends from roughly amino acid 112-118 of CG to
25 residue 145 at the C-terminus. The precise length of CTP in the constructs herein will be clear from the content.

In the fusions described herein, native CTP can be used or a "variant" thereof. By "variant" is meant a conservative analog of the peptide residues from about
30 112-118 to 145, i.e. this sequence wherein about 1-5 amino acids of the sequence are altered without substantial change in properties. Often this variation results simply from mutation to obtain appropriate restriction sites.

Although it is recognized that glycosylation pattern has a profound influence on activity both qualitatively and quantitatively, for convenience the terms FSH, LH, TSH, and CG beta subunits refers to the amino acid sequence characteristic of the peptides, as does "alpha subunit". When only the beta chain is referred to, the terms will be, for example, FSH beta; when the heterodimer is referred to, the simple term "FSH" will be used. It will be clear from the context in what manner the glycosylation pattern is affected by, for example, recombinant expression host or alteration in the glycosylation sites. Forms of the glycoprotein with specified glycosylation patterns will be so noted.

As used herein, the alpha subunit "minigene" refers to the gene construction disclosed herein in the description of the construction of pM^2/CG alpha or pM^2/alpha . This "minigene" is characterized by retention only of the intron sequence between exon III and exon IV, all upstream introns having been deleted. In the particular construction described, the N-terminal coding sequences which are derived from exon II and a portion of exon III are supplied from cDNA and are ligated directly through an XbaI restriction site into the coding sequence of exon III so that the introns between exons I and II and between exons II and III are absent. However, the intron between exons III and IV as well as the signals 3' of the coding sequence are retained. The resulting minigene can conveniently be inserted as a BamHI/BglII segment. Other means for construction of a comparable minigene are, of course, possible and the definition is not restricted to the particular construction wherein the coding sequences are ligated through an XbaI site. However, this is a convenient means for the construction of the gene, and there is no particular advantage to other approaches, such

as synthetic or partially synthetic preparation of the gene. The definition includes those coding sequences for the alpha subunit which retain the intron between exons III and IV but no other introns.

5 A "transfected" recombinant host cell, i.e., a cell "transfected" with the recombinant expression systems of the invention, refers to a host cell which has been altered to contain this expression system by any convenient manner of introducing it, including
10 transfection, viral infection, and so forth. "Transfected" refers to cells containing this expression system whether the system is integrated into the chromosome or is extrachromosomal. The "transfected" cells may either be stable with respect to inclusion of the expres-
15 sion system or not. In short, "transfected" recombinant host cells with the expression system of the invention refers to cells which include this expression system as a result of their manipulation to include it, when they natively do not, regardless of the manner of effecting
20 this incorporation.

"Expression system" refers to a DNA sequence which includes a coding sequence to be expressed and those accompanying control DNA sequences necessary to effect the expression of the coding sequence. Typically, these
25 controls include a promoter, termination regulating sequences, and, in some cases, an operator or other mechanism to regulate expression. The control sequences are those which are designed to be functional in a particular target recombinant host cell and therefore the
30 host cell must be chosen so as to be compatible with the control sequences in the constructed expression system.

As used herein "cells", "cell cultures", and "cell lines" are used interchangeably without particular attention to nuances of meaning. Where the distinction between them is important, it will be clear from the context. Where any can be meant, all are intended to be included.

Isolation of the Gene Encoding FSH Beta

An important aspect of the present invention is the provision of an expression system for FSH beta-encoding DNA which provides beta-FSH that readily dimerizes to form the hormone. The gene, suitable for inclusion in expression systems intended for host cells capable of processing introns, was prepared as follows:

Genomic DNA from JAr choriocarcinoma cells (a human placental donor) was partially digested with MboI and cloned into the BamHI site of lambda MG3, a vector described Helms, C., et al. DNA (1985) 4:39-49; this vector is a derivative of lambda L47 which is described by Loenen, W.A.M., et al. Gene (1980) 10:249-259. The size of the inserts was typically 15-20 kb. Approximately 5×10^5 plaques were obtained and screened according to the method of Benton, W.D., et al. Science (1977) 196:180-182 using the 41 mer encoding amino acids 94-107 of exon III of human FSH beta as described by Watkins, P.C., et al. DNA (1987) 6:205-212. This 41 mer has the sequence:

TGTACTGTGCGAGGCCTGGGGCCCAGCTACTGCTCCTTTGG.

Two positive clones were isolated by repeated plaque purification and shown by restriction analysis to be identical; furthermore, the PstI cleavage patterns were consistent with those obtained by Glaser, T. et al. Nature (1986) 321:882-887 (supra). Restriction fragments were subcloned into pUC18 for further restriction analysis and into M13 for sequencing by the dideoxy chain termination method of Sanger, F., Proc Natl Acad Sci USA (1977) 74:5463-5467. A 3.7 kb HindIII/BamHI fragment contained

in the 16.5 kb insert of these clones contains the hFSH beta coding sequence.

The clones were designated lambdaI and lambdaR, and have identical restriction maps and are approximately
5 16.5 kb in length. The restriction map of the full length clones are shown in Figure 1, along with a restriction map of the 3.7 kb human FSH beta coding sequence.

The results of sequencing the human FSH beta gene are shown in Figure 2. As shown in Figure 2, the
10 coding sequence is divided into three exons. Exon I contains a 5' untranslated tract previously reported to encode two transcripts of either 33 or 63 bp (Jameson, J.L. et al. Mol Endocrinol (1988) 2:806-815). Exon II encodes an 18 amino acid signal peptide and amino acids 1-
15 35 of the mature protein. Exon III encodes amino acids 36-111 and about 1.1 kb of 3' untranslated sequence. Exons I and II are separated by an intron of about 800 bp, and exons II and III by an intron of about 1.4 kb.

The nucleotide sequence obtained is similar to
20 that reported by Watkins, T.C. et al. DNA (1987) 6:205-212 and Jameson, J.L. et al. (supra), except that tyrosine 58 is encoded by TAC rather than TAT and there are differences from Watkins in the 3' and 5' untranslated regions. A putative transcriptional start site 32 bp downstream
25 from the TATA element is assigned by analogy to the bovine gene reported by Kim, K.E., et al., DNA (1988) 7:227-333. The sequence in Figure 2 shows a single polyadenylation signal (AATAAA) overlapping the termination codon and recent evidence from the bovine gene (supra) and human
30 clones (Jameson, J.L. et al., (supra)) indicates the presence of an approximately 1.1 kb 3' untranslated tract which may contain alternate polyadenylation signals.

The amino acid sequence shown in Figure 2 is identical to that reported by that of Watkins (supra) but
35 differs from that reported earlier by protein sequencing of purified human FSH beta. The carboxy terminal sequence

Tyr-Pro-Thr-Ala-Leu-Ser-Tyr reported by Saxena, D.B., J Biol Chem (1976) 251:993-1005 is found neither in the sequence shown in Figure 2 nor in the protein based sequence reported by Shome, B., et al., J Clin Endocrinol Metab (1974) 39:203-205. A more recent determination of the amino acid sequence confirms the sequence deduced from the DNA (Stone, B. et al. J Prot Chem (1988) 7:325-339.

An important modification of the beta-FSH chain encoding DNA is obtained when the 34 amino acid carboxy terminal peptide (CTP) of the chorionic gonadotropin beta chain is fused to the C-terminus. In this form of the hormone, the C-terminal Glu of FSH beta at position 111 is extended with the amino acid sequence representing from about amino acids 112-118 to 145 of the beta CG sequence.

Construction of Expression Vectors for Native Human Alpha Subunit and its Muteins

The alpha subunit used in the constructions of the invention is advantageously in the form of the minigene as described above. It has been found that the use of the minigene enhances the production of the alpha subunit as well as its capacity to dimerize with the appropriate beta subunit to form the desired heterodimer. The minigene or the other forms of DNA encoding the alpha subunit can also be modified to provide alpha subunit muteins. Some of these are particularly useful as antagonists, such as those having deletions or alterations in amino acids at positions 88-92 of the native sequence. In addition, the gene encoding the alpha subunit can be modified so as to provide glycosylation mutants.

It is understood in the art that N-linked glycosylation occurs at the tripeptide site Asn-X-Thr/Ser, two of which sites occur in the human alpha subunit, at Asn52 and Asn78. Site-directed mutagenesis was performed on a human alpha subunit fusion gene to alter these sites, wherein the fusion gene was constructed as follows:

The alpha subunit cDNA is obtained as described by Matzuk, M.M. et al. J Cell Biol (1988) 106:1049-1058, incorporated herein by reference (supra) as a BamHI/XhoI framed nucleotide sequence containing an XbaI site in the coding sequence. A genomic fragment bounded by EcoRI and a XhoI site containing exons III and IV, with an XbaI site in exon III was obtained from the human choriocarcinoma library. XbaI/XhoI digestion of both the genomic fragment and alpha subunit cDNA, followed by religation at the XbaI site creates the alpha subunit minigene as a BamHI/XhoI fragment, containing a BglII site derived from the genomic fragment downstream of exon IV. The reconstructed gene contains two coding regions (exon II and III and exon IV), a single intron, and the polyadenylation signals 3' to exon IV. The BamHI/BglII fragment digested from the minigene is used as the alpha subunit-encoding insert in the construction of expression vectors. The use of the alpha subunit minigene gives impressive and surprising results in affecting the efficiency of expression systems for production of the dimeric hormones.

In constructing genes encoding muteins of the alpha subunit, the BamHI/XhoI fragment itself is ligated into M13 UM20 for site-directed mutagenesis. For alteration of Asn52 and Asn78, respectively, the 22-mer oligomers GGTGACGTCCTTTTGCACCAAC and CTTAGTGGAGCGGGATATG respectively were used. This resulted in a substitution of aspartate residues for asparagine. Three mutants were constructed: alpha(Asn1) (position 52), alpha(Asn2) (position 78), and alpha(Asn1+2) (both positions). Corresponding changes were made by substituting the codon for alanine in place of that for threonine at positions 54 and 80 using the 26-mers: GTGGACTCTGAGGCCACGTTCTTTTG and CAGTGGCAGCCGCATGGTTCTCCAC, respectively to obtain alpha(Thr1), alpha(Thr2) and alpha(Thr1+2).

35

The wild type or mutant alpha subunits were then ligated into the host vector pM² as the 2.4 kb minigenes as BamHI/BglII segments so as to be placed under control of the LTR promoter by insertion into the BamHI site downstream of the LTR, as shown in Figure 3. pM², as described by Matzuk, M.M. et al., Proc Natl Acad Sci USA (1987) 84:6354-6358, is a derivative of pSV2Neo and contains the ampicillin resistance gene (amp^r), the neomycin resistance gene (neo^r), and the Harvey murine sarcoma virus long terminal repeat (LTR) promoter with a unique downstream BamHI site. pM²/alpha contains an alpha subunit minigene downstream from a second LTR. The resulting vector shown, pM²/CG alpha, can be used to express the alpha subunit per se or can be used as the source of the human alpha expression unit for construction of a host vector which can also accommodate the expression of an additional DNA. One example of useful additional DNA comprises those encoding the various beta hormone chains.

The host vector pM²/alpha may be prepared by excising this alpha expression unit from pM²/CG alpha as an EcoRI/EcoRI fragment and ligating it into the EcoRI site of pM² (Matzuk, M.M. et al. Mol Endocrinol (1988) 2:95-100) incorporated herein by reference and also shown in Figure 3. As therein described, the BamHI site at the 5' end of the gene is first deleted, and the XhoI site at the 3' end of the gene is converted to an EcoRI site using oligonucleotide linkers. This vector can then also accommodate a DNA encoding a beta subunit.

In addition to muteins of the alpha subunit which have altered glycosylation patterns, a group of muteins with reduced or zero activity in signal transduction is also prepared. Experiments using chemical derivatization in in vitro assays indicate that amino acids at positions 88-92 (tyr-tyr-his-lys-ser) are necessary for the signal transduction activity of the hormone.

Accordingly, deletion or alteration of one or more of these amino acids by site-directed mutagenesis results in analogs which continue to bind to receptor but have reduced or negligible activity. All four of the hormones sharing this alpha subunit can thus be prepared as antagonists for the relevant hormone.

Both the wild type and mutant vectors can be used as a source of human alpha subunit, and provide host expression systems for recombinant production of the dimeric hormones. Of particular importance are mutants of the alpha subunit in which the glycosylation site at Asn-52 is altered. Such mutated sequences when ligated into expression systems and transfected into appropriate host cells result in production of proteins which, when combined with the appropriate beta subunit have antagonist activity for the relevant hormone.

The foregoing constructions are, of course, merely illustrative of expression vectors or systems which can be constructed for the production of alpha subunits or the corresponding heterodimeric hormones. Alternate control sequences including, for example, different promoters, can be ligated to the coding sequence of human beta subunits or the alpha minigene to effect expression in other eucaryotic cells which will provide suitable glycosylation. A variety of control sequences is known in the art, and methods to ligate the beta subunits or alpha minigene coding sequence are of course also available. For example, suitable yeast promoters include promoters for synthesis of glycolytic enzymes including those for 3-phosphoglycerate kinase, or promoters from the enolase gene or the leu2 gene. Suitable mammalian promoters include the early and late promoters from SV40, or other viral promoters such as those derived from polyoma,

adenovirus 2, bovine papilloma virus or avian sarcoma viruses. Suitable viral and mammalian enhancers can also be used. Expression in insect cells using a baculovirus promoter has also been reported. While less common,
5 expression systems suitable for plant cells are also available.

Construction of Vectors for the Beta Subunits

With respect to the coding DNA for beta
10 subunits, such as FSH-beta or LH-beta, or their modified forms, genomic DNA or modified genomic DNA can be inserted directly into expression systems intended for eucaryotic host cells capable of processing introns. The nucleic acid sequences encoding the protein can be used directly
15 from the genomic clone or cDNA clone as described herein, or can be entirely or partially synthesized using standard solid phase oligonucleotide synthesis techniques as described, for example, by Nambiar, K.P. et al. Science (1984) 223:1299 or by Jaye, E. et al. J Biol Chem (1984)
20 259:6311. These techniques are now commercially available. It is evident, of course, that not only the specific native nucleotide sequences can be employed, but also nucleotide sequences employing codons which are degenerate with these, as well as allelic forms.

25

Construction of Expression Vectors for FSH

To obtain satisfactory expression of FSH, one useful expression construct results from insertion of the beta subunit into a host vector in a position so as to be
30 under control of an appropriate promoter wherein the host vector includes an expression system for the alpha minigene, such as pm^2/α . When inserted as the HindIII/BamHI fragment, the FSH will be operably linked to the LTR promoter of its expression system. In an illustrative construction, the 5' HindIII site of FSH beta-
35 containing pUC18 vector (pFSH beta), was converted to a

BglII site using oligonucleotide linkers, and the modified pFSH beta vector digested with BglII and BamHI. The resulting 3.7 kb BglII/BamHI fragment was inserted into the unique BamHI site downstream of the LTR promoter which
5 lacks a tissue-specific element so as to be operably linked to said promoter, and orientation was confirmed by restriction analysis.

An important mutein-encoding sequence of FSH beta is obtained by ligating the DNA sequence encoding the
10 carboxy terminal extension peptide of CG beta (CTP) to the 3' end of the FSH beta encoding sequence. To the C-terminal Glu of FSH beta at position 111 is ligated the downstream sequence of from about amino acid 112 to 118 to the carboxy terminal amino acid 145 of CG beta, or a
15 variant thereof. Preferred variants include those wherein the Ser at position 118 of CG beta is replaced by Ala. This extended form is conveniently obtained by ligation of HindIII-digested FSH beta encoding insert with the HindIII digest of CG beta cDNA, where this religation results in
20 the Ser ---> Ala substitution. The protein resulting from expression of this sequence when produced as the heterodimer FSH is expected to have the biological activity of native FSH but a prolonged circulating half-life. This expectation is made in view of the longer half-life
25 of CG as compared to LH, which is ascribable to the presence of extensive O-linked glycosylation in the CTP region as described by Van Hall, E. Endocrinol (1971) 88:456. A major problem with FSH in clinical use is the relatively short circulating half-life of this protein (Wide, L. Acta
30 Endocrinol (1986) 112:336)...

Thus, "extended" forms of FSH beta are those wherein the approximately 28-34 amino acid sequence represented by residues 112-118 to 145 of human CG (or a variant thereof) is ligated to the C-terminus of FSH-beta.

As stated above, "variant" is meant a conservative analog of the peptide residues from about 112-118 to 145, i.e. this sequence wherein about 1-5 amino acids of the sequence are altered without substantial change in properties.

The construction of one embodiment of this extended form of FSH is outlined in Figure 4. As shown, the coding sequence for human FSH beta for the C-terminal portion located in the third exon of the human FSH beta gene is mutated in the stop codon to obtain a HindIII site. Similarly, the region encoding the beginning of the CTP region of the human CG beta subunit, also in the third exon of the gene, is mutated at positions 116 and 117 to obtain a corresponding HindIII site. This mutation converts the Asp residue at position 116 to glutamic and the serine residue at position 117 to Ala. The mutated genomic fragments are then digested with HindIII and BamHI and ligated as shown to obtain a fragment encoding the extended FSH beta; if desired further mutagenesis is performed to convert the Ala residue to a hydrophilic Ser residue. The FSH beta subunit, thus contains the region of beta CG at positions 118-145. The BamHI/BamHI fragment containing the modified beta FSH encoding sequence is then ligated into the BamHI site of pM^2 under control of the LTR promoter.

When cotransformed into Chinese hamster ovary cells along with the expression vector for the alpha subunit pM^2/α , yields of secreted FSH of approximately $1 \text{ mg}/10^6 \text{ cells}/24 \text{ hr}$ cultured in 1L of medium are obtained.

Additional muteins of FSH beta are prepared by deleting or altering the N-linked glycosylation sites represented by the Asn-Thr combinations at positions 7/9 and 24/26 of the native sequence. The protein produced from expression of a system capable of expressing the

genes encoding these muteins is expected to show adequate receptor binding with respect to the FSH beta receptor, and when heterodimerized with a suitable alpha subunit can be used as an antagonist for FSH activity; or, when
5 heterodimerized with a normal alpha subunit can be used as an FSH substitute.

Construction of Expression Vectors for Human LH Muteins

10 Difficulties have been encountered in the art in the production of LH using recombinant techniques because the LH beta chain, for example, compared to the chorionic gonadotropin (CG) beta chain, fails to dimerize efficiently to the alpha subunit and is not secreted when
15 obtained as the monomer. The mutein LH beta chains of the invention do not suffer from these disadvantages.

The effect of various alterations of the LH encoding sequence on secretion of the LH chain alone or on dimerization and secretion of the intact hormone can be
20 determined by expressing the LH-encoding sequence in cells transformed with a suitable vector containing an expression system encoding LH beta alone or in cells which coexpress the alpha subunit. In the latter case, the alpha subunit expression system can either be supplied on
25 a separate vector, such as pM²/CG alpha, or can be supplied on a vector which also contains the beta subunit. This can be done, for example, by insertion of the gene encoding the beta subunit into the host vector pM²/alpha described above.

30 Figure 5 shows the differences in amino acid sequence between the beta chains of LH and CG in humans. A reading frame shift after the codon for position 114 in LH as compared to CG leads to the translation of a hydrophobic 7 amino acid sequence which forms the C-terminus of
35 LH contrasted with the relatively hydrophilic 31 amino acid sequence which provides the C-terminal peptide (CTP)

of CG in this case, residues 115-145. In the muteins of the invention, the 7 amino acid hydrophobic C-terminus of LH is deleted or replaced with a hydrophobic sequence. In addition, one or more substitutions are made for the amino acids which inhibit secretion or dimerization. These LH amino acids are selected from the group consisting of the tryptophan residue at position 8 (trp⁸), the isoleucine residue at position 15 (ile¹⁵), the methionine residue at position 42 (met⁴²) and the aspartic acid residue at 77 (asp⁷⁷). The combination of these modifications results in beta LH subunits which are easily secreted. In addition, the combination of the deletion or replacement of the 7 carboxy terminal amino acid sequence in combination with at least one substitution for an amino acid selected from the group consisting of trp⁸, ile¹⁵, met⁴² and the threonine at position 58 (thr⁵⁸) enhances dimer formation when the alpha subunit is coexpressed. Other substitutions may also be made, but the combination of the alteration or deletion of the 7 amino acid terminus with the alteration of one of the above-specified amino acids is necessary to have the desired effect.

The construction of genes encoding these modified forms of LH will permit the construction of cell lines to produce the desired hormone analog for the provision of quantities of the analog useful for therapeutic and/or diagnostic purposes.

As stated in the Background section, the DNA sequence encoding the beta LH chain is readily available. In the illustration below, the wild type LH-encoding starting material was provided as a 1270 bp HindIII/BamHI fragment containing the coding sequences of exons II and III of the LH beta gene.

As further described below, in two preferred embodiments, the 7 amino acid sequence at the C-terminus of LH can either be deleted directly by insertion of a stop codon after the codon for amino acid 114, or can be

replaced by a hydrophilic sequence, preferably the CTP from human CG, or a variant thereof. The replacements for the additional interfering amino acids at the above-referenced positions are those which destroy the anti-secretion or anti-dimerization effects of these amino acids. Table 1 shows illustrative modified forms of the beta subunit which were prepared by mutagenesis after insertion of the LH beta gene into M13mp19 and hybridization with the appropriate oligonucleotides. After the mutagenesis, the modified HindIII/BamHI fragment was ligated to the sequence of exon I to provide the LH beta fragment as a 1400 bp BglII/BamHI fragment. However, in variants where the gene contained the CTP region of the CG sequence, the BglII/BamHI fragment contained 3600 bp.

The construction of the genes encoding the various LH muteins and LH/CG chimeras is outlined in Figure 6. Expression systems for both chimeras of LH and CG beta subunits and muteins were engineered and tested for the ability to produce subunits which are readily secreted and/or from dimers. As shown in Figure 6, the chimeras are constructed by using the appropriate portions of the LH beta or CG beta encoding genes. The chimeras are noted by LC or CL, depending on which of the subunits forms the N-terminus and which the C-terminus, and the number following the designation designates the number of the last amino acid in the beta subunit which forms the N-terminus. Thus, LCbeta41 shown in Figure 6 produces a beta subunit which contains amino acids 1-41 of human LH beta subunit as its N-terminus and amino acids 42-145 of human CG beta subunit at its C-terminus. As the beta subunit is understood, it is deleted from the designation in the text. CL87 designates a construct which contains amino acids 1-87 of CG followed by amino acids 88-121 of LH beta. Constructs with deleted C-terminal sequences beyond position 114 are designated delta T in Figure 6 and (1-114) in the table. Thus, for example, LC87 (1-114)

contains amino acids 1-87 of human LH beta subunit and amino acids 88-114 of human CG beta. Specific mutations are indicated by the amino acid substituted for that in the native subunit superscripted by the position at which the mutation occurs. Thus, LH thr⁴² indicates the human LH beta subunit which has the methionine at position 42 replaced by threonine. Combinations of alterations in the sequence are correspondingly indicated; for example, LH gly⁴⁷ala⁵¹ (1-114) is a human beta LH subunit wherein the naturally-occurring amino acids at positions 47 and 51 are replaced by gly and ala respectively, and the portion of LH beta represented by amino acids 115-121 is deleted.

Expression vectors were constructed using pM² for the illustrative mutants in Table 1 by insertion of the genes into the BamHI site downstream of the LTR promoter. The vectors were then transfected alone or along with pM²/CG alpha (supra) into Chinese hamster ovary cells, as described by Matzuk, M.M., et al., Proc Natl Acad Sci USA (1987) 84:6354-6358; Matzuk, M.M., et al., J Cell Biol (1988) 106:1049-1059, both cited above. Successful transformants were selected in 0.25 ug/ml G418, and expression was detected by immunoprecipitation of metabolically labeled cells to select monomer- and dimer-secreting cell lines.

Stably transfected CHO cell lines were maintained in "medium-1" (Ham's F12 medium supplemented with penicillin (100 U/ml), streptomycin (100 ug/ml), and glutamine (2 mM)) containing 5% of v/v fetal calf serum on 0.125 mg/ml G418 in a humidified 5% CO₂ incubator.

The cells were plated at 300,000-350,000 cells per well into 12-well dishes in 1 ml medium 1 supplemented with 5% fetal calf serum 1 day prior to labeling. For continuous labeling, cells were washed twice with cysteine-free "medium-2" (medium-1 supplemented with 5% dialyzed calf serum) and labeled for 6 hours in 1 ml of cysteine-free medium 2 containing 20 uCi/ml labeled

cysteine. For pulse chase experiments, the cells were washed twice and preincubated for 1.5 hours in cysteine-free medium-2, followed by a 20 minute labeling in cysteine-free medium-2 containing 100 uCi/ml labeled

5 cysteine, then washed twice with medium 2 containing 1 mM unlabeled cysteine and incubated in the unlabeled medium.

The medium and cell lysates were prepared, immunoprecipitated, and treated as described by Corless, C.L., et al., J Cell Biol (1987) 104:1173-1181, and in the
10 Matzuk PNAS paper cited above. Antisera against CG beta, LH beta, and the alpha subunit were prepared by standard methods; antisera generated against CG beta cross-reacts fully with LH beta and was used to detect LH beta as well. For characterization of the immunoprecipitates on SDS
15 gels, 15% SDS polyacrylamide gels were soaked for 10 minutes in 1 M sodium salicylate, dried, and autoradiographed with preflash film, and scanned, if desired, with an LKB Ultrascan XL laser densitometer.

The effects of the crucial alterations are shown
20 in Table 1.

Table 1

Secretion and Assembly of
LH Beta-CG Beta Chimeras and Muteins

Beta Subunit	Monomer		Dimer	
	t1/2 (h)	Recovery ⁺ (%)	t1/2 (h)	Recovery ⁺ (%)
CG (beta)	1.9	>95	1.2	>95
30 LH (beta)	10	<20	5.5	42
LHthr ^{15*}	7.4	40	7.7	30
LH(1-114)	7	40	6	80
35 CG(1-114)	2.6	91	2	90

	LC41	5	85	1	>95
	CL41	6.6	50	2.5	50
	LC87(1-114)	>10	25	5.5	32
5	CL87	5.3	85	3	45
	CL87(1-114)	2.3	95	1.6	>95
	CL41(1-114)	2.5	>95	1	94*
<hr/>					
10	LHthr ¹⁵ (1-114)*	4.2	83	3.1>	95**/40*
	LHarg ⁸	>10	25	5.5	32
	LHarg ⁸ (1-114)	5.1	91	2.0	91
	LHarg ⁸ arg ¹⁰ (1-114)	5.2	90	2.3	95
	CL41thr ¹⁵ (1-114)	2.5	>95	-	-
15	LC41thr ¹⁵	4.2	91	-	-
<hr/>					
	LHthr ⁴² (1-114)	6.0	80	2.6	84
	LHgly ⁴⁷ ala ⁵¹ (1-114)	7.5	28	-	-
20	LHasn ⁵⁸ (1-114)	6.9	40	2.2	>95
	LHasn ⁷⁷ (1-114)	5.7	70	-	-
	LHtyr ⁸² ala ⁸³	10	50	-	-

25 + For mutants which are slowly secreted, recovery is estimated by comparing the amount secreted and the amount which has disappeared from the lysate in 10 hours.

* Monoglycosylated form recovered in the medium since alpha obscures proper quantitation.

** Recovery of di-glycosylated form is estimated.

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35

As shown in the table, as compared to the behavior of native LH beta, either the deletion of the 7-amino acid C-terminal sequence or a critical single amino acid alteration has some noticeable effect, both on monomer secretion and recovery and on dimer secretion and recovery, but generally the effects are intermediate to a combination of the C-terminal deletion with the appropriate amino acid substitution. Using monomer secretion as a criterion, for example, the $t_{1/2}$ decreases dramatically from 10 hours and the recovery increases significantly from a value of less than 20% when the C-terminal deletion is combined with the substitution of the hydrophobic amino acid isoleucine at position 15, of tryptophan at position 8, the methionine at position 42 or the aspartic acid residue at position 77 (although this effect is not as great). Similar results are obtained in assessing the dimer secretion, except that the alteration of the threonine residue at position 58 appears to be very effective in regard to dimerization, while having little impact on the secretion of monomer, and the alteration in the aspartic to asparagine at position 77 appears to have no effect. The data obtained for the chimeras constructed by combinations of LH and CG are consistent with these results.

The modified forms of LH beta of the invention are thus capable of enhanced dimerization with alpha subunit and secretion from the host cell; these modifications to the beta subunit make it possible to produce LH in practical amounts using recombinant host cells. The desirable properties are a result of the deletion or modification of the hydrophobic C-terminus of the beta chain at positions 115-121, in combination with an amino acid modification at a specified location. Any one of the

hydrophobic residues at positions 8, 15, and 42 may be replaced by a hydrophilic residue; alternatively, the threonine residue at position 58 may be replaced by asparagine.

5 Suitable hydrophilic amino acid residues include aspartic, glutamic, glycine, histidine, lysine, asparagine, glutamine, arginine, serine, and threonine. Especially preferred are the neutral hydrophilic amino acids glycine, asparagine, glutamine, serine, and
10 threonine. Especially preferred are replacement of trp at position 8 with arginine and ile at position 15 or met at position 42 with threonine, i.e., with the residue at the corresponding position in CG beta subunit.

 With respect to the hydrophobic 7-amino acid
15 sequence at the C-terminus, modification is conveniently made by deletion of the sequence, generally by insertion of a stop codon at position 115 in the gene. Alternatively, the sequence may be replaced by a suitable hydrophilic sequence, most conveniently the 31-amino acid
20 sequence representing the carboxy terminal peptide at positions 115-145 of the human chorionic gonadotropin beta subunit, or a variant thereof.

 Of course, additional amino acid modifications which do not interfere with the activity of the LH hormone
25 may also be included. Specifically, modification of amino acids at positions corresponding to amino acid differences between the LH sequence and the CG sequence, whereby the appropriate residue from CG replaces that of LH, are preferred, where such additional substitutions are limited
30 to 1-2 residues.

Production of Hormones with Glycosylation Defined by Host Choice

 The expression systems constructed according to
35 the preceding paragraphs can be transfected into a designated host, so that the protein obtained has a

glycosylation pattern which is internally consistent within the recovered protein and which is characteristic of the recombinant host employed. This glycosylation may also be optionally modified by changes in the glycosylation sites contained on the amino acid sequence. Recombinant hosts suitable to the expression system constructed must, of course, be employed.

With respect to the illustrated expression system employing the Harvey murine sarcoma virus long terminal repeat, suitable host cells are mammalian, in particular, host cells which are derived from rodents. A particularly preferred host cell, because of convenience and consistency in glycosylation pattern, is a Chinese hamster ovary cell. For the illustrated expression systems, transfectants of CHO cells were obtained by transfection of CHO cells according to the procedure of Matzuk, M.M. et al. (1987), supra, except that the cells were maintained in alpha MEM containing 10% (v/v) bovine calf serum, 100 U/ml penicillin and 100 ug/ml streptomycin as a growth medium. Stable transfectants were selected from this growth medium supplemented with 250 ug/ml G418. Dimer-secreting clones are isolated by screening media and lysates with alpha and beta antisera. The human alpha subunit can be expressed on a separate vector or on the same vector as the beta subunit. For example, pM²/alpha or pM²CG/alpha can be cotransfected with the plasmid containing the beta subunit encoding DNA, or pM²/alpha into which the DNA encoding the beta subunit has been inserted can be used.

By way of illustration, the expression systems described above for human FSH beta inserted into pM² for expression of FSH beta alone or into pM²/alpha for expression in tandem with the alpha subunit were transfected into CHO cells and stable clones shown to express the beta subunit or dimer were continuously labeled with ³⁵S-cysteine for 6 hr. The proteins secreted into the media

and from cell lysates were immunoprecipitated with appropriate antisera and resolved on SDS-PAGE. The results are shown in Figure 7 in comparison with the behavior of transformants expressing the gene for human CG beta.

5 Figure 7a, which displays gels from 6 hr labeling, shows that in the absence of the alpha subunit, FSH beta is retained in the lysate, while, as shown in Figure 7b, when the alpha subunit is present, the dimer is formed and efficiently secreted into the medium. The results of
10 experiments wherein the cells are pulse labeled with ^{35}S -cysteine for 20 min and chased with unlabeled cysteine for up to 12 hr are shown in the remaining segments of Figure 7. Figure 7c shows the results for the beta subunit of CG where the lower molecular weight beta subunit in the
15 medium is apparently due to the differences in the extent of glycosylation at the 2 Asn-linked glycosylation sites on CG beta and is unique to this beta subunit. The half-life of CG beta from lysates and of appearance of CG beta in the medium are identical at about 2 hr and almost all
20 the secreted beta subunit can be recovered.

 Figure 7d shows that FSH beta alone is secreted much less efficiently and as does CG beta, disappears from the cell lysates after about 5 hr; less than 20% is recovered in the medium after 12 hr. Similarly to the beta
25 subunits of LH and TSH, FSH beta alone is inefficiently secreted and slowly degraded intracellularly. However, Figure 6e shows that the presence of the alpha subunit stabilizes and enhances the secretion of the beta subunit for FSH. The half-life for disappearance from the lysates
30 was about 90 min, and 90% was recovered in the medium after 12 hr. This behavior is similar to that shown for TSH above, but different from both CG and LH.

 The transformants secreting dimer were tested for biological activity and by chromatofocusing. Rat
35 granulosa cells were treated with increasing aliquots (0.01-1.0 ul/ml) of recombinant FSH-containing medium in

an in vitro assay for steroidogenesis as described by Jia, X.C., et al. J Clin Endocrinol Metab (1986) 62:1243-1249; Jia, X.C. Endocrinol (1986) 119:1570-1577. The results of this assay are shown in Figure 8. These results show that maximum estrogen production was 10-fold higher than basal values and similar to that induced by pituitary FSH standard LER-907. Neither recombinant CG nor purified FSH beta alone stimulate estrogen production. The results show that the biologically active FSH dimer is secreted at about 1.1 ± 0.4 IU/ 10^6 cells/24 hr corresponding to a specific activity of 6600 IU/mg immunoreactive FSH. The cells thus secrete 500 ng FSH/ 10^6 cells in 24 hr.

The medium from the transfected CHO cell cultures was chromatographed on a PBE-94 column with a pH gradient from 7.0-3.5 and the FSH bioactivity in each fraction was determined based on the in vitro assay described above. As a control, purified human FSH (NIADD-hFSH-1-3) was treated similarly. The results, shown in Figure 9, indicate that both recombinant and isolated human FSH show one major peak of activity with pI values between 5.0-3.6 for recombinant FSH and between 5.2 and 3.6 for purified FSH. Pituitary FSH displayed a heterogeneous range of bioactive alkaline forms which was not seen in the recombinant protein. The results from chromatofocusing clearly indicate a uniform nonheterogeneous glycosylated form of the protein.

In addition, a mutant CHO cell line deficient in N-acetyl glucosaminyltransferase I activity, such as 15B, can be used to alter glycosylation in both the alpha and beta subunits of FSH of the heterodimeric hormones. It has been shown that FSH produced in CHO cells deficient in the glycosylation enzyme N-acetyl-glucosamine transferase-1 (NAGT-) results in an Asn-linked (GLcNAc)₂ (mannose)₅ oligosaccharides. Production of FSH in CHO cells lacking CMP-sialic acid transport into the Golgi apparatus (ST⁻) results in sialic acid deficient FSH.

Influence of Glycosylation on Secretion of Human Alpha Subunit

The resultant alpha subunit expression systems constructed as described in the paragraphs above were transfected into CHO cells using a modification of the calcium phosphate method wherein cells were selected for insertion of the plasmid DNA by growing in a culture medium containing 0.25 mg/ml of G418. Resistant colonies were harvested eleven days after transfection and screened for expression of alpha subunit by immunoprecipitation of media or lysates of the cells with the appropriate antiserum. The CHO cells were maintained in Ham's F12 medium supplemented with pen/strep and glutamine (2 mM) containing 5% v/v FCS at 37°C in a humidified 5% CO₂ incubator; transfected clones were maintained with the addition of 0.125 mg/ml G418.

For metabolic labeling, on day 0 the cells were placed into 12 well dishes at 350,000 cells/well in 1 ml medium supplemented with 5% FCS. For continuous labeling experiments, the cells were washed twice with cysteine-free medium supplemented with 5% dialyzed calf serum in place of FCS and were labeled for 7-8 hr in 1 ml of cysteine-free medium containing 5% dialyzed calf serum and 50 uCi/ml ³⁵S-cysteine (more than 1,000 Ci/mmol). The cell lysates or media were then immunoprecipitated and, if appropriate, treated with endoglycosidases as described by Corless, C.L. et al. J Cell Biol (1987) 104:1173-1181. The immunoprecipitates were resolved on 15% SDS polyacrylamide gels.

Using this analysis method, it was clear that the level of glycosylation had an influence not only on the secretion of the alpha subunit, but also on its resultant molecular weight. The results are summarized in Table 2:

Table 2

	Lysate	Medium	% Secreted
alpha WT .	23 kd	28 kd	> 95%
5 alpha(Asn1) or alpha(Thr1)	20 kd	22 kd	> 95%
alpha(Asn2) or alpha(Thr2)	20 kd	23.5 kd	< 20%
alpha(Asn 1+2) or			
alpha(Thr 1+2)	15 kd	15 kd	~ 50%
alpha WT + tunicamycin	15 kd	15 kd	> 95%

10

As shown in Table 2, loss of the glycosylation at the position 78 Asn glycosylation site resulted in a substantial decrease in the efficiency of secretion.

15 Evidently additional carbohydrate processing takes place during the secretion which is manifested in the higher molecular weight form found in the medium. This was confirmed by treatment of the secreted forms with endoglycosidaseF which cleaves complex oligosaccharides in

20 addition to high mannose noncomplex and hybrid-type oligosaccharides. More than 95% of the secreted material is sensitive to endoglycosidaseF, but not to endoglycosidaseH which cleaves only high mannose noncomplex and hybrid-type oligosaccharides.

25 Pulse chase experiments performed as described in Matzuk, M.M. et al. J Cell Biol (1988) 106:1049-1059, incorporated herein by reference, shows that the somewhat lower levels of secreted alpha(Asn1) or alpha(Thr1) is due to clonal variation rather than differences in secretion

30 or degradation rates. However, the mutants lacking glycosylation at the second (position 78) glycosylation site showed decreased secretion rates and an increased degradation rate.

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It is clear from these results that the glycosylation at position 2 has a profound influence both on secretion rate and on the intracellular stability of the alpha subunit alone.

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Influence of Alpha Subunit Glycosylation on Secretion of hCG

The influence of the glycosylation state of the alpha subunit on the efficiency of assembly of the dimeric hormone hCG was also studied in Matzuk, M.M. (supra).

10 In the clones wherein hCG beta is formed in excess of the alpha subunit, all of the wild type alpha subunit is mobilized into the dimeric form of the hormone for secretion. On the other hand, those mutants which are
15 missing oligosaccharide from position 52 (glycosylation site 1) are deficient in the secretion of intact hCG dimer by virtue of altering the assembly and/or stability of the dimer complex. However, loss of glycosylation at position 2 seems to have less effect on assembly of the dimeric
20 hormone. Removal of both glycosylation sites has an intermediate effect on assembly; the removal of glycosylation from both sites seems to have a lesser effect on the ability of the hormone to assemble than removal of the glycosylation from position 1 alone. In
25 addition, the beta subunit of hCG stabilizes the mutants at position 2 from degradation of the alpha subunit.

It is clear from the foregoing results that the glycosylation pattern of the alpha subunit determines both the ability of the alpha subunit itself to be secreted and
30 its ability to dimerize with the beta subunit to form intact hormone.

As noted in the paragraph describing the production of alpha subunit muteins, certain designated amino acids in the carboxy-terminal portion of the alpha subunit
35 are required for signal transduction activity. Accordingly, inactivated alpha subunit is useful in the

construction of antagonists by dimerization with the appropriate beta subunit of any of the hormones FSH, LH, CG and TSH.

However, it is clear that the influence of the glycoprotein alpha subunit on secretion of beta subunits of the four hormones in this group differs depending on the nature of the beta subunit. Matzuk, M.M., et al. Molec Endocrinol (1988) 2:95-100, incorporated herein by reference, show that the presence of the alpha glycoprotein has a different effect on the secretion of human thyrotropin as opposed to human CG or LH. It has been shown that in the absence of the alpha subunit, CG beta is efficiently secreted, but TSH and LH beta subunits are slowly degraded intracellularly and less than 10% secreted into the medium. However, in the presence of the alpha subunit, CG beta is also secreted efficiently as the intact dimeric hormone while only 50% of LH beta appears in the medium as LH dimer. On the other hand, the alpha subunit efficiently combines with TSH beta, since greater than 95% of this beta subunit was secreted as the dimer. This demonstrates that the assembly of the dimeric hormone is dependent on the nature of both subunits.

As described in the paragraphs with regard to the construction of expression systems for FSH beta, mutein forms of FSH beta which are superior in circulating half-life can be produced by construction of a mutein containing the CTP amino acid sequence at the carboxy terminus of human CG beta. In addition, the N-linked glycosylation sites of the FSH beta subunit can be deleted or altered without affecting receptor binding activity.

Mutein forms of hCG are also included in the scope of the invention. Various muteins of hCG containing deleted or altered N-linked glycosylation sites are recombinantly produced by construction of expression systems analogous to those for FSH beta and for the alpha subunit from the suitably modified forms of the appropri-

ate genes. Absence of any or all of the hCG N-linked oligosaccharides had only a minor effect on receptor affinity; with respect to the production of cAMP and steroidogenesis, absence of N-linked oligosaccharides from CG beta or from Asn-78 of the alpha subunit had no effect. However, the oligosaccharide at asparagine-52 of alpha was critical for cAMP and steroid production. In addition, its absence unmasked differences in the two N-linked oligosaccharides present in CG beta and inhibited in vitro biological activity.

Effect of Glycosylation on Biological Properties

It has been demonstrated that complete deglycosylation of human chorionic gonadotropin results in a hormone which retains its ability to bind to receptor, but is no longer capable of effecting the ordinary biological response of the cell on which the receptor is borne. Similar effects of complete deglycosylation are obtained with the additional reproductive hormones LH and FSH. Accordingly, the unglycosylated mutants of the invention, whether obtained through recombinant production in a host which is incapable of such glycosylation, or by mutation so as to eliminate the N-linked glycosylation sites, provides antagonists useful in situations where reproductive function must be regulated and an antagonist is required. Partial deglycosylation as would be obtained, for example, by providing a deglycosylated form of the alpha subunit in combination with a beta subunit, provides molecules with intermediate effects. The alteration of glycosylation sites in the alpha subunit has been described above. Similar alteration of the glycosylation pattern in the beta subunits can be achieved by mutation to eliminate one or both of the glycosylation sites at

positions 15 and 30 of the CG beta subunit; one or both of the glycosylation sites at position 7 and 24 of the FSH beta subunit or the glycosylation site at position 30 of the LH beta subunit.

5 These alternative forms of the hormones can conveniently be produced by the methods of the invention, such as the use of expression systems using the alpha minigene as described above or in combination with the additional modifications of the FSH beta and CG beta
10 subunits, also as described above.

Effect of CTP on Clearance

 The invention further provides a method to improve the clearance characteristics of protein pharmaceuticals and hormones in general by extending their biological half-lives in vivo through a modification which comprises ligation of the carboxy terminal portion of the HCG beta subunit or a variant thereof, as above-defined, to the carboxy terminus of the pharmaceutical or hormone.
15 Such modifications can be made in a manner analogous to that described for the construction of a gene encoding the extended FSH beta subunit above and inclusion of the modified gene in any suitable expression system for production of the protein. Recombinantly produced protein pharmaceuticals are now produced commercially, and accordingly,
20 expression of the modified gene can be effected using commonly known techniques. Suitable candidates for modification include the hormones such as insulin (in which the A subunit would preferably be extended), human growth
25 hormone, bovine growth hormone, tissue plasminogen activator, erythropoietin, various hormone regulators such as LHRH and its analogs, and, in general, any peptide or protein administered for its biological effect. In
30 general, this modification results in an extended half-life, which is advantageous in permitting lower dosages to
35 be used.

Utility and Administration

5 The hormones and other pharmaceuticals of the
present invention are formulated for administration using
methods generally understood in the art. Typical formula-
tions and modes of administration are described in
10 Remington's Pharmaceutical Sciences, Mack Publishing Co.,
Easton, PA, latest edition. These formulations are
typically for systemic administration, such as by injec-
tion, but oral formulations or topical formulations may
15 also be employed.

 The choice of formulation, mode of administra-
tion, and dosage level are dependent on the particular
hormone or protein and can be optimized for the appropri-
15 ate indication using generally recognized techniques.

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Claims

1. A modified LH beta subunit having enhanced ability to dimerize with alpha subunit and enhanced secretion as a dimer from mammalian recombinant host cells, as compared to wild-type LH beta subunit, wherein said subunit is a modified wild-type beta subunit wherein the 7-amino acid hydrophobic sequence at positions 115-121 is deleted or replaced by a hydrophilic sequence, and at least one residue selected from the group consisting of trp⁸, ile¹⁵, and met⁴² is replaced by a hydrophilic amino acid or thr⁵⁸ is replaced by asn.
2. The beta subunit of claim 1 wherein said residues 115-121 are deleted.
3. The subunit of claim 1 wherein residues 115-121 are replaced by the carboxy terminal peptide (CTP) represented by residues 115-145 of human chorionic gonadotropin (hCG).
4. The subunit of claim 1 wherein the tryptophan residue at position 8 is replaced by an arginine residue.
5. The subunit of claim 1 wherein the isoleucine residue at position at position 15 is replaced by a threonine residue.
6. The subunit of claim 1 wherein the methionine residue at position 42 is replaced by a threonine residue.
7. The subunit of claim 1 wherein the threonine residue at position 58 is replaced by an asparagine residue.

8. A DNA sequence encoding the modified LH beta subunit of claim 1.

5 9. An expression system capable, when contained in compatible host cells, of expressing a DNA sequence encoding the modified LH beta subunit of claim 1, said expression system comprising a DNA encoding said LH beta subunit operably linked to compatible control sequences.

10 10. Recombinant host cells transfected or transformed with the expression system of claim 9.

15 11. A method to produce a modified LH beta subunit which method comprises culturing the cells of claim 10 under conditions wherein said DNA encoding said subunit is expressed; and recovering the LH beta subunits produced.

20 12. The expression system of claim 9 which further contains an expression system capable of producing the human glycoprotein alpha subunit.

25 13. The expression system of claim 12 wherein the expression system for said alpha subunit contains the gene encoding the alpha subunit as a minigene.

30 14. Recombinant host cells transfected or transformed with the expression system of claim 12.

35 15. The recombinant host cells of claim 10 which further contain an expression system capable of producing the human glycoprotein alpha subunit.

16. The cells of claim 14 wherein the expression system for said alpha subunit contains the gene encoding the alpha subunit as a minigene.

5 17. A method to produce a human LH dimer containing a modified beta subunit which comprises culturing the cells of claim 14 under conditions wherein the expression system capable of producing the alpha subunit and the modified LH beta subunit are operable; and
10 recovering the human LH produced.

 18. A method to produce a human LH dimer containing a modified beta subunit which method comprises culturing the cells of claim 15 under conditions wherein
15 the expression system capable of producing the alpha subunit and the modified LH beta subunit are operable; and recovering the human LH produced.

 19. A human LH dimer containing, as the beta
20 subunit, the modified LH beta subunit of claim 1.

 20. A human LH dimer produced by the method of claim 17.

25 21. A human LH dimer produced by the method of claim 18.

 22. A recombinant expression system capable, when transformed into a recombinant host cell, of expressing the gene encoding the human glycoprotein alpha
30 subunit; said expression system comprising a gene encoding said subunit operably linked to compatible control sequences, wherein said gene is a minigene.

35 23. Recombinant host cells transformed or transfected with the expression system of claim 22.

24. A method to produce human alpha subunit
which comprises culturing the cells of claim 23 under
conditions wherein the gene encoding the human alpha
5 subunit is expressed and
recovering human alpha subunit from the cell
culture.

25. The expression system of claim 22 which
10 further includes an expression system capable of express-
ing the gene encoding the beta subunit of a human
glycoprotein hormone selected from the group consisting of
FSH, LH, TSH and CG.

26. Recombinant host cells transformed or
15 transfected with the expression system of claim 25.

27. A method to produce a human glycoprotein
hormone selected from the group consisting of FSH, LH, TSH
20 and CG which method comprises culturing the cells of claim
26 under conditions wherein the genes encoding the alpha
subunit and beta subunit are expressed and
recovering the human glycoprotein hormone from
the cell culture.

28. Recombinant host cells of claim 23 which
are further transformed with an expression system capable
of expressing the gene encoding the beta subunit of a hu-
man glycoprotein hormone selected from the group consist-
30 ing of FSH, LH, TSH and CG.

35

29. A method to produce a human glycoprotein hormone selected from the group consisting of FSH, LH, TSH and CG which comprises culturing the cells of claim 28 under conditions wherein the genes encoding the human
5 alpha and beta subunits are expressed and recovering the hormone from the cell culture.

30. A human glycoprotein hormone selected from the group consisting of FSH, LH, TSH and CG having a
10 glycosylation pattern characteristic of said hormone prepared by the method of claim 27.

31. A human glycoprotein hormone selected from the group consisting of FSH, LH, TSH and CG having a
15 glycosylation pattern characteristic of said hormone prepared by the method of claim 29.

32. A mutein of the human glycoprotein alpha subunit which mutein lacks a glycosylation site at position 52 or 78 or both and/or which mutein lacks one or
20 more of amino acids 88-92 of the native subunit.

33. A recombinant expression system capable, when transformed into a compatible recombinant host cell,
25 of expressing a gene encoding the mutein of the human glycoprotein alpha subunit of claim 32.

34. Recombinant host cells transformed or transfected with the expression system of claim 33.
30

35. A method of producing an alpha subunit mutein which method comprises culturing the cells of claim 34 under conditions wherein the gene encoding such said mutein is expressed and
35 recovering the alpha subunit from the cell culture.

36. The recombinant expression system of claim
33 which further includes an expression system capable of
expressing the gene encoding a beta subunit of a human
glycoprotein hormone selected from the group consisting of
FSH, LH, TSH and CG.

37. Recombinant host cells transformed or
transfected with the expression system of claim 36.

38. A method to produce a human glycoprotein
hormone selected from the group consisting of FSH, LH, TSH
and CG which method comprises culturing the cells of claim
37 under conditions wherein the genes encoding the alpha
and beta subunits are expressed and
recovering the hormone from the cell culture.

39. The recombinant host cells of claim 34 which
further include an expression system capable of expressing
the gene encoding the beta subunit of a human glycoprotein
hormone selected from the group of FSH, LH, TSH and CG.

40. A method to produce a human glycoprotein
hormone selected from the group consisting of FSH, LH, TSH
and CG which method comprises culturing the cells of claim
39 under conditions wherein the genes encoding the alpha
and beta subunits are expressed and
recovering the hormone from the cell culture.

41. A human glycoprotein hormone selected from
the group consisting of FSH, LH, TSH and CG having a
glycosylation pattern characteristic of said hormone
prepared by the method of claim 38.

42. A human glycoprotein hormone selected from the group consisting of FSH, LH, TSH and CG having a glycosylation pattern characteristic of said hormone prepared by the method of claim 40.

5

43. A modified alpha subunit produced by the method of claim 35.

44. An extended human FSH subunit wherein the amino acid sequence of a carboxy terminal peptide (CTP) representing positions from about 112-118 to 145 of the human CG beta subunit or a variant form thereof is appended to the C-terminal amino acid residue at position 111 of the wild-type FSH beta subunit.

10
15

45. A recombinant DNA sequence in a purified and isolated form which encodes the extended FSH beta subunit of claim 44.

46. An expression system capable, when contained in compatible host cells, of expressing a DNA sequence encoding the extended FSH beta subunit of claim 44 said expression system comprising a DNA encoding said subunit operably linked to compatible control sequences.

20
25

47. Recombinant host cells transfected or transformed with the expression system of claim 46.

48. A method to produce extended human FSH beta subunit which method comprises culturing the cells of claim 45 under conditions favoring expression of the encoding DNA and

30

recovering the extended FSH beta subunit from cell culture.

35

49. The expression system of claim 46 which further contains an expression system capable of producing the human glycoprotein alpha subunit.

5 50. Recombinant host cells transformed or transfected with the expression system of claim 49.

51. A method of producing a modified human FSH which method comprises culturing the cells of claim 50
10 under conditions wherein the genes encoding said alpha and beta subunits are expressed, and
recovering the FSH from the cell culture.

52. The recombinant host cells of claim 47
15 which further include an expression system capable of expressing a gene encoding the human glycoprotein alpha subunit.

53. A method to produce a modified human FSH
20 which comprises culturing the cells of claim 52 under conditions wherein the genes encoding the alpha and beta subunits are expressed and
recovering the modified FSH from the cell
culture.

25 54. A modified human FSH containing as the beta subunit, the extended beta subunit of claim 44.

55. A modified human FSH produced by the method
30 of claim 51.

56. A modified human FSH produced by the method
of 53.

35

57. A pharmaceutical composition useful in treating reproductive disorders in human subjects which comprises the LH glycoprotein hormone of claim 19 optionally in admixture with an additional glycoprotein hormone
5 selected from the group consisting of FSH and CG in admixture with a suitable pharmaceutically acceptable excipient.

58. A pharmaceutical composition useful in
10 treating reproductive disorders in human subjects which comprises the glycoprotein hormone of claim 30 optionally and different from said glycoprotein hormone of claim 40 in admixture with an additional glycoprotein hormone
15 selected from the group consisting of FSH, LH and CG and different from said glycoprotein hormone of claim 50 in admixture with a suitable pharmaceutically acceptable excipient.

59. A pharmaceutical composition useful in
20 treating reproductive disorders in human subjects which comprises the glycoprotein hormone of claim 41 optionally and different from said glycoprotein hormone of claim 47 in admixture with an additional glycoprotein hormone
25 selected from the group consisting of FSH, LH and CG in admixture with a suitable pharmaceutically acceptable excipient.

60. A pharmaceutical composition useful in
30 treating reproductive disorders in human subjects which comprises the FSH glycoprotein hormone of claim 54 optionally in admixture with an additional glycoprotein hormone
selected from the group consisting of LH and CG in
admixture with a suitable pharmaceutically acceptable
excipient.

61. A modified human glycoprotein hormone selected from the group consisting of FSH, LH and CG wherein the alpha subunit is a mutein which lacks a glycosylation site at position 52 or 78 or both and/or
5 which mutein lacks one or more of amino acids 88-92 of the native subunit.

62. A human LH dimer which contains a modified beta subunit wherein said subunit is a modified wild-type
10 beta subunit wherein the 7-amino acid hydrophobic sequence at positions 115-121 is deleted or replaced by a hydrophilic sequence, and at least one residue selected from the group consisting of trp⁸, ile¹⁵, and met⁴² is replaced by a hydrophilic amino acid or thr⁵⁸ is replaced
15 by asn, or asp⁷⁷ is replaced by asn.

63. A human FSH dimer which contains an extended beta subunit wherein the amino acid sequence of a carboxy terminal protein CTP representing positions from
20 about 112-118 to 145 of the human CG beta subunit or a variant form thereof is appended to the C-terminal amino acid residue at position 111 of the wild-type FSH beta subunit.

25 64. A pharmaceutical composition useful in treating reproductive disorders in human subjects which comprises the glycoprotein hormone of claim 61 optionally in admixture with an additional glycoprotein hormone selected from the group consisting of FSH, LH and CG, and
30 different from said glycoprotein hormone of claim 61, in admixture with a suitable pharmaceutically acceptable excipient.

65. A pharmaceutical composition useful in treating reproductive disorders in human subjects which comprises the glycoprotein hormone of claim 62 optionally in admixture with an additional glycoprotein hormone
5 selected from the group consisting of FSH and CG in admixture with a suitable pharmaceutically acceptable excipient.

66. A pharmaceutical composition useful in
10 treating reproductive disorders in human subjects which comprises the glycoprotein hormone of claim 63 optionally in admixture with an additional glycoprotein hormone selected from the group consisting of LH and CG in
15 admixture with a suitable pharmaceutically acceptable excipient.

67. A modified protein or peptide pharmaceutical, wherein said modification comprises extension of the carboxy terminus of said protein or peptide with an amino
20 acid sequence of the carboxy terminal portion of human CG beta subunit or a variant thereof.

68. The modified pharmaceutical of claim 67 wherein the protein or peptide is a hormone.
25

69. The modified pharmaceutical of claim 68 wherein the hormone is selected from the group consisting of human growth hormone, bovine growth hormone, FSH and LH.
30

35

1/10

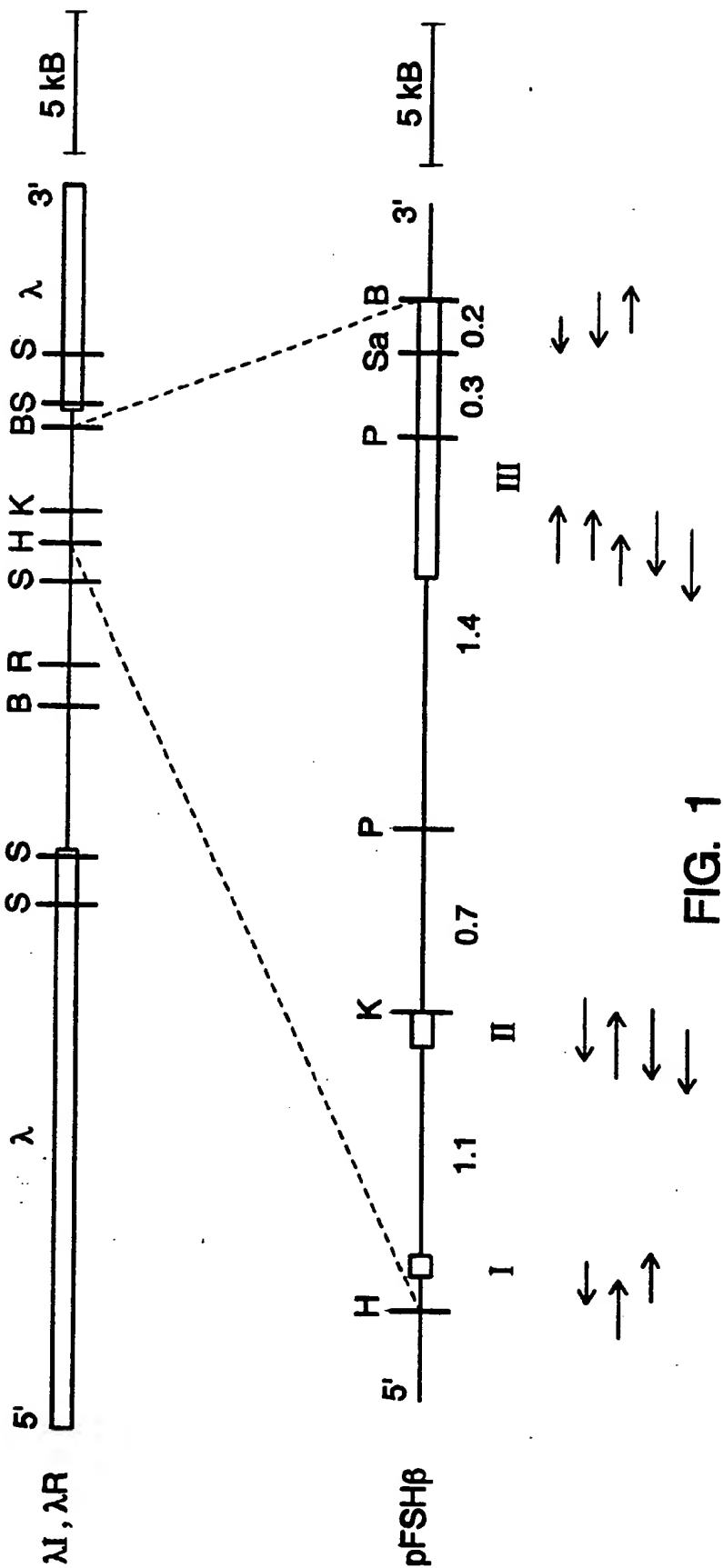


FIG. 1

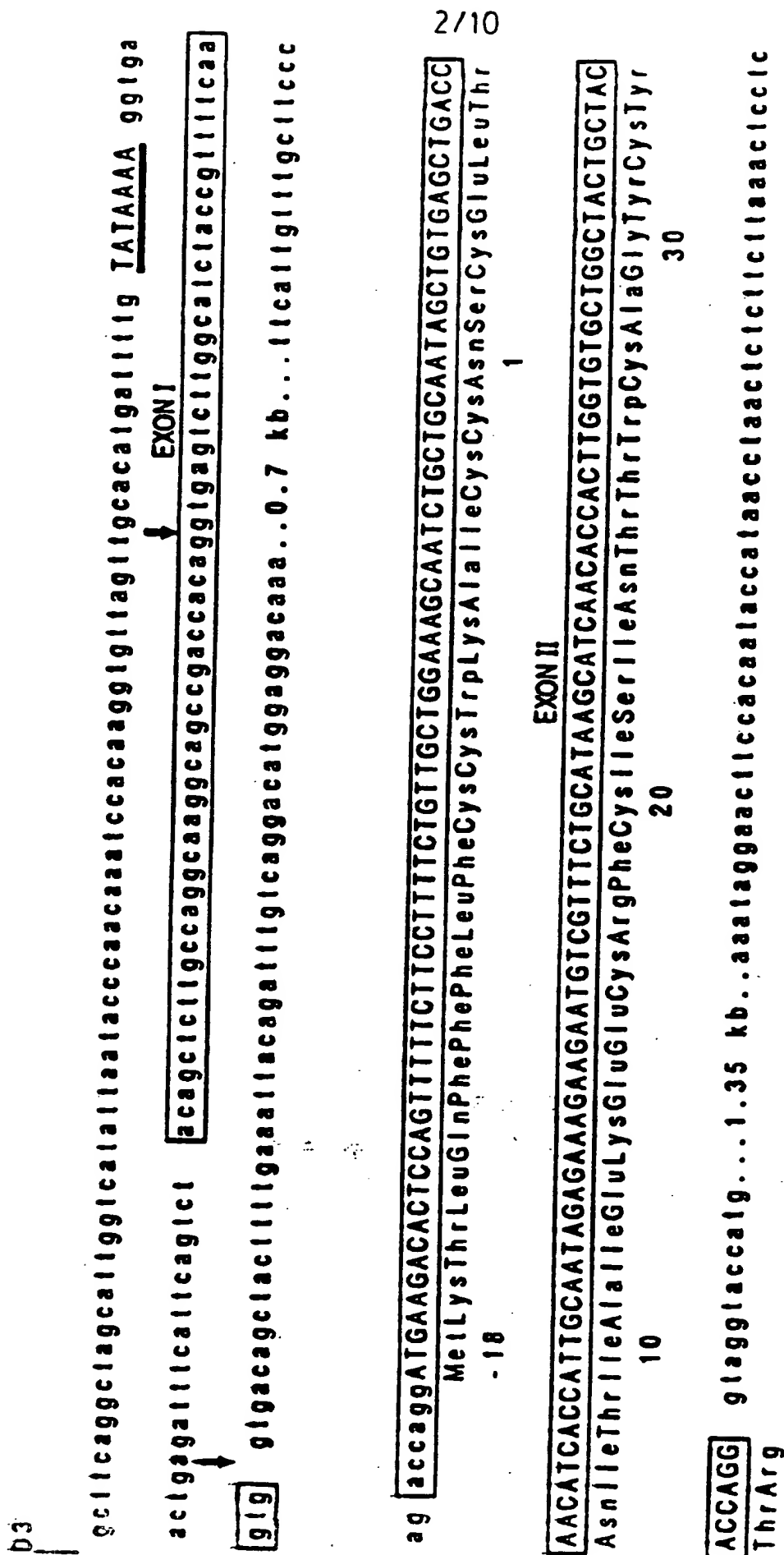


FIG. 2A

4 / 10

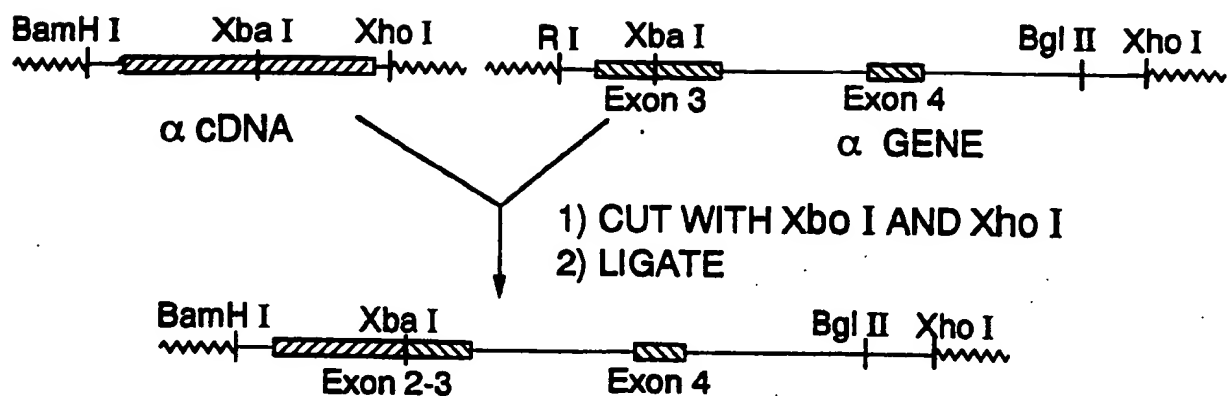


FIG. 3A

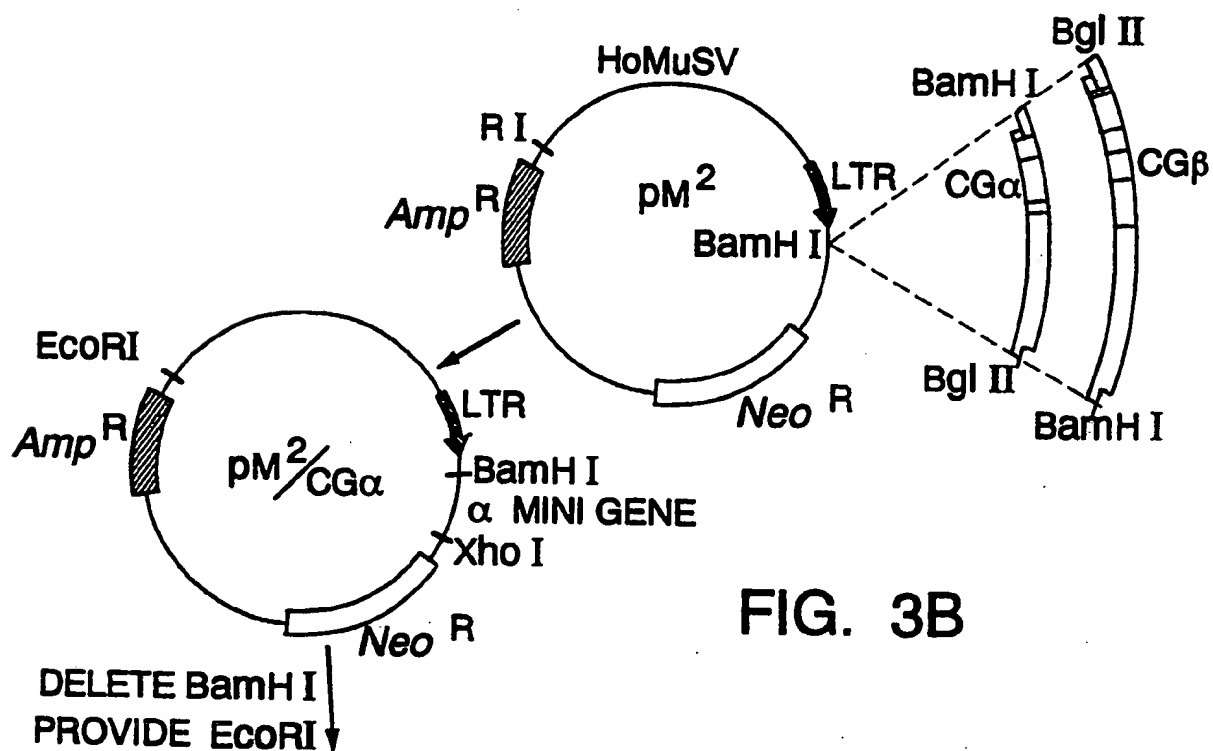
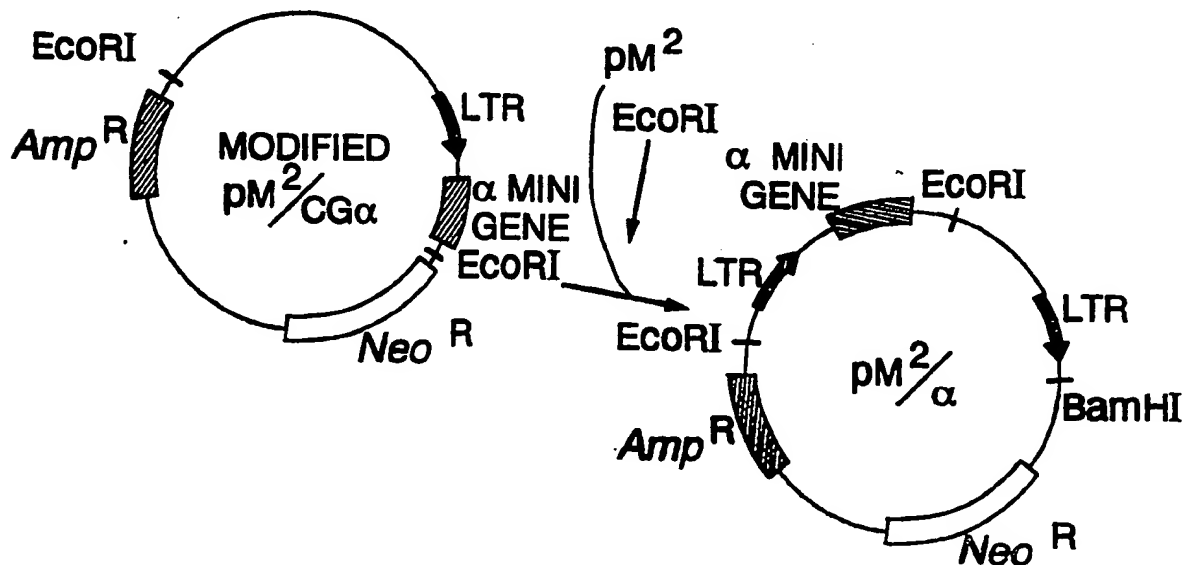


FIG. 3B



SUBSTITUTE SHEET

5/10

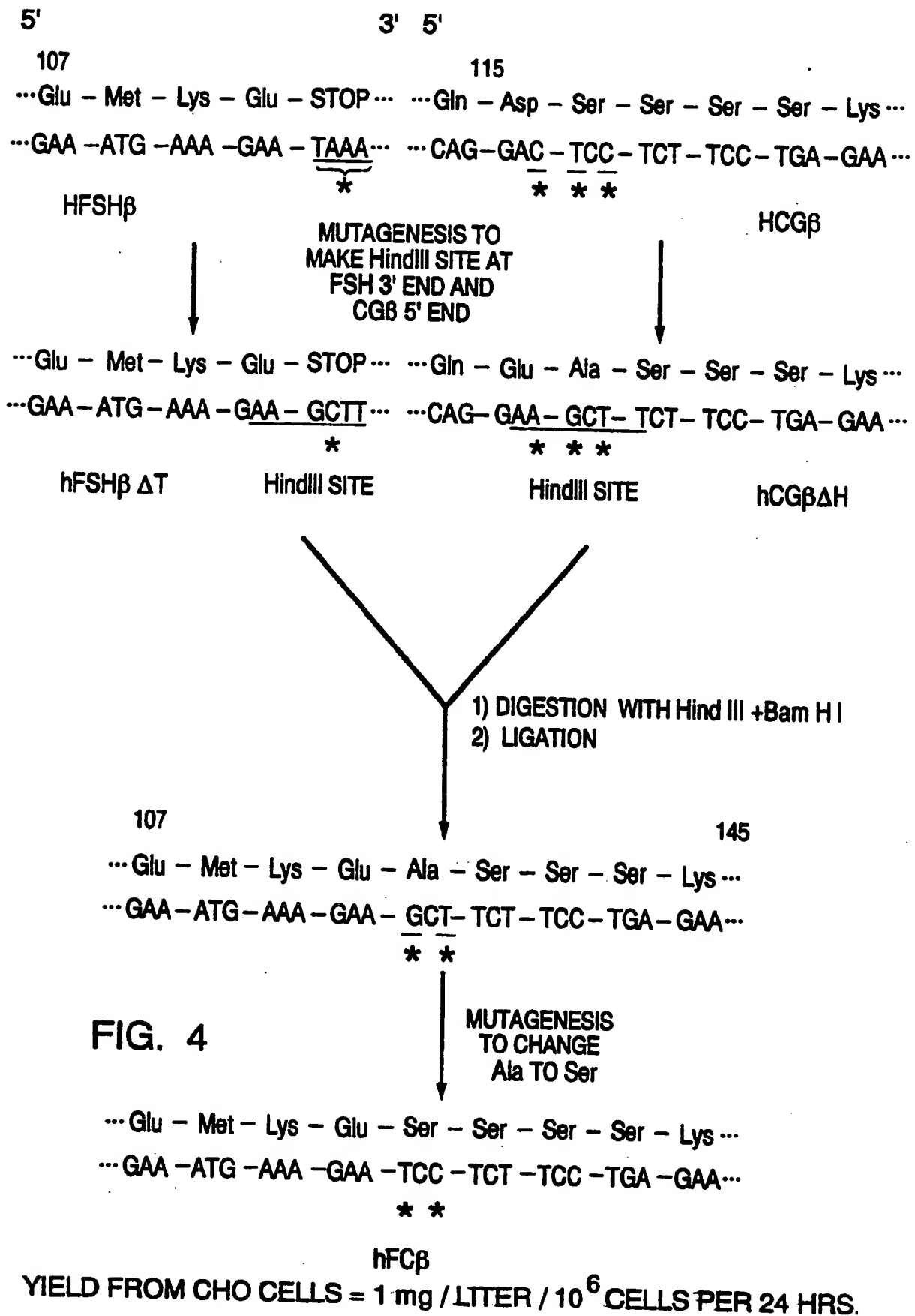


FIG. 4

5
E/G.

AA DIFF:	8	42	89	112	115-121
	10	47	91	114	115-145

	1	2	8	9	10	13	15	23	26	30	34	38	42
LH	ARG	-	-	-	TRP	C	HIS	-	ASN	-	ILE	-	-
CG	LYS	-	-	-	ARG	C	ARG	-	ASN	-	THR	-	-

- 1) 8 TRP → ARG: LH8
2) 8 TRP → ARG: 10 HIS → ARG: LH8/10
3) 15 ILE → THR: LH 15 *
4) 42 MET → THR: LH 42
5) 47 ALA → GLY, 51 PRO → ALA: LH47/51
6) 58 THR → ASN: LH58
7) 77 ASP → ASN: LH 77
8) 82 PHE → TYR, 83 PRO → ALA: LH 82/83

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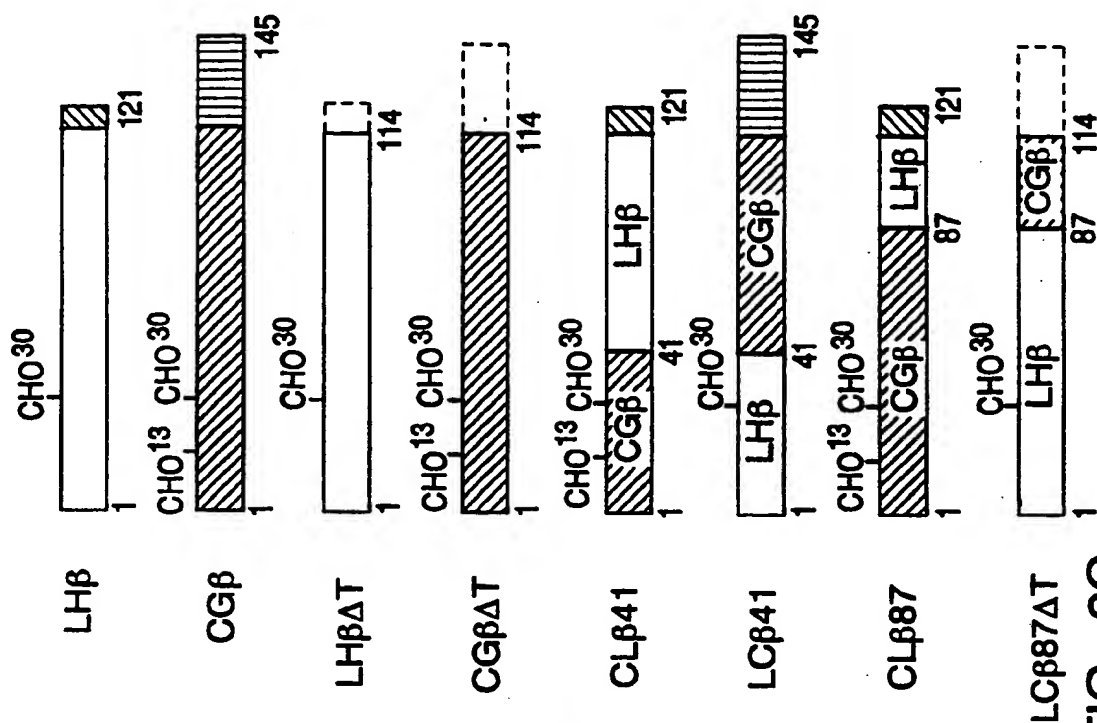


FIG. 6C

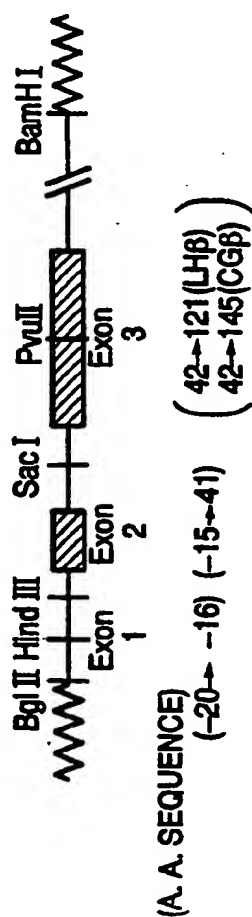


FIG. 6A

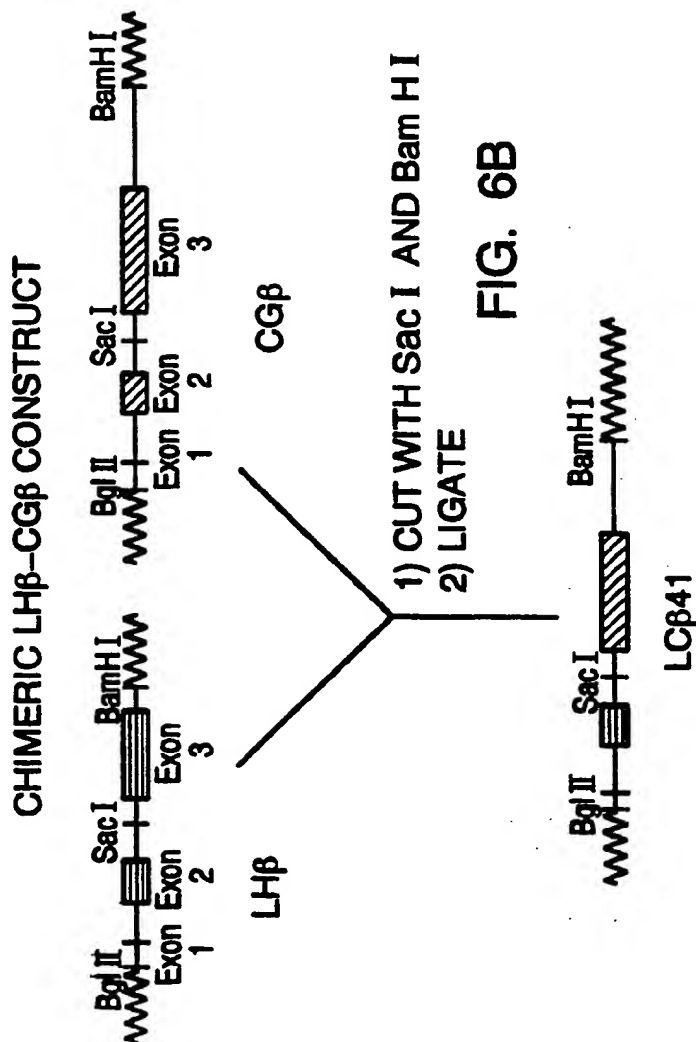


Fig. 6B

CG β LYSATE MEDIUM
0 1 2 4 6 12 | 1 2 4 6 12

FIG. 7C

FSH β LYSATE MEDIUM
0 1 2 4 6 12 | 1 2 4 6 12

FIG. 7D

FSH LYSATE MEDIUM
0 1 2 4 6 12 | 1 2 4 6 12

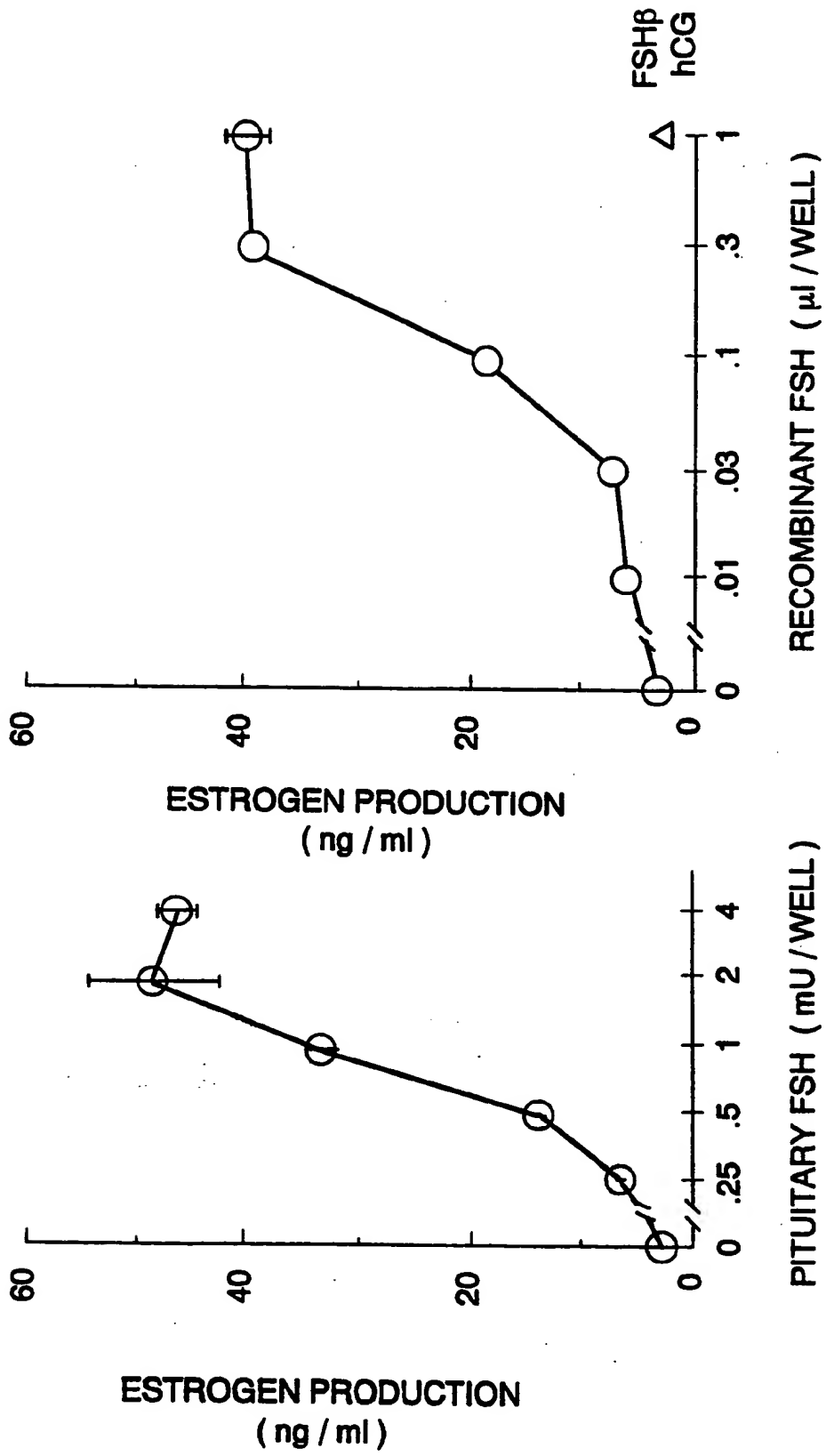
FIG. 7E

FSH β FSH Dimer
 $\alpha^M \beta^L$ | $\alpha^L \beta^M$

-30
-21.5

1 2 3 4 5 6 7 8

FIG. 7A FIG. 7B



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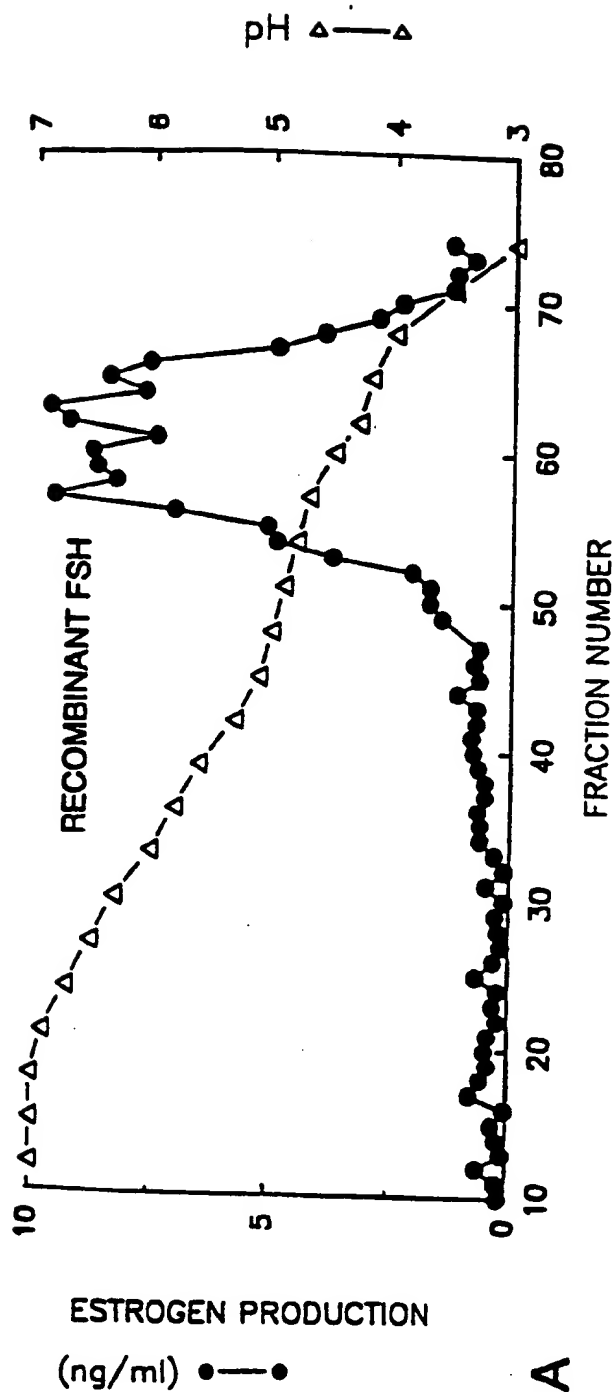


FIG. 9A

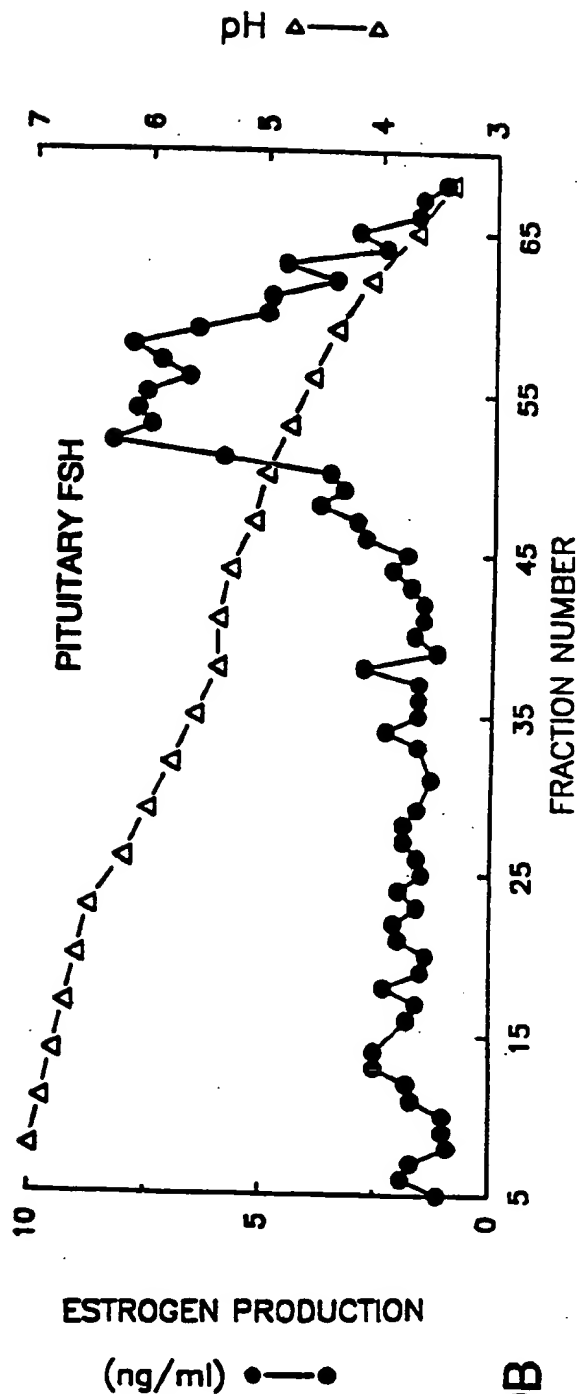


FIG. 9B

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/01037

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 3

According to International Patent Classification (IPC) or to both National Classification and IPC
 IPC (5): C12P 21/00, C12P 21/02, C07K 7/00, A61K 37/38
 U.S. CL: 435/69.4, 69.7, 70.1; 530/395,397,398

II. FIELDS SEARCHED

Minimum Documentation Searched 4

Classification System

Classification Symbols

U.S. 435/69.4, 69.7, 70.1
 530/395,397,398

Documentation Searched other than Minimum Documentation
 to the Extent that such Documents are Included in the Fields Searched 5

USPIO- Automated Patent System-USEPAT, JMEMO, JPOAES. Dialog Files-BIOSIS PREVIEWS, CLAIMS
 INPADOC/FAMILY AND LEGAL STATUS, WORLD PATENTS INDEX, CHINESE
 PATENT ABS IN ENGLISH, BIOTECHNOLOGY ABSTRACTS

Search terms: see attachment

III. DOCUMENTS CONSIDERED TO BE RELEVANT 14

Category 6	Citation of Document, 14 with indication, where appropriate, of the relevant passages 17	Relevant to Claim No. 14
Y	US, A, 4,746,508 (Carey et al.) 24 May 1988	57-60, 64-69
Y	WO, A, 85/01958 (Reddy et al.) 09 May 1985. Note the abstract and pages 2-3.	57-60, 64-69
Y	WO, A, 85/01959 (Reddy et al.) 09 May 1985. Note the abstract and pages 2-3.	57-60, 64-69
T	US, A, 4,923,805 (Reddy et al.) 08 May 1985. Note col. 9-10	57-60, 64-69
Y	US, A, 4,302,386 (Stevens) 24 November 1981. Entire document.	57-60, 64-69
Y	J. Molec. Appl. Genet. vol.1, No. 1, 1981. (Fiddes et al.), "The gene encoding the common alpha subunit of the four human glycoprotein hormones". p 3-18. Entire Document.	57-60, 64-69
Y	Nature. vol. 286, 14 August 1980. (Fiddes et al.), "The cDNA for the B-subunit of human chorionic gonadotropin suggests evolution of a gene by readthrough into the 3'-untranslated region". p 684-687. See page 684.	57-60, 64-69
Y	WO, A, 86/04589 (Reddy et al.) 14 August 1986. Note the abstract and at least page 14 lines 14-21.	57-60, 64-69

* Special categories of cited documents: 15

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"Z" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search 2

25 JUNE 1990

Date of Mailing of this International Search Report 3

30 JUL 1990

International Searching Authority 1

ISA/US

Signature of Authorized Officer 20

CHRISTOPHER S.F. LOW

III. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of Document	Relevant to Claim No
Y	FEBS Letters. vol. 188. No.2, September 1985. (Hayashizaki <u>et al.</u>), "Molecular cloning of the human thyrotropin-B subunit gene" p 394-400. See especially p 394 and Fig. 3.	57-60, 64-69
Y	Molec. Endocrinol. vol. 2. 1988. (Jameson <u>et al.</u>) "Human follicle-stimulating hormone B-subunit gene encodes multiple messenger ribonucleic acids". p 806-815. Entire document.	57-60, 64-69
Y	Proc. Natl. Acad. Sci. USA. vol. 84., September 1987. (Matzuk <u>et al.</u>), "Effects of preventing O-glycosylation in the secretion of human chorionic gonadotropin in Chinese hamster ovary cells". p 6354-6358. Entire document.	57-60, 64-69
Y	DNA. vol. 6., No.3, 1987 (Watkins <u>et al.</u>), DNA sequence and regional assignment of the human follicle-stimulating hormone B-subunit gene to the short arm of human chromosome 11. p 205-212. Entire Document.	57-60, 64-69
Y	<u>Frontiers in Thyroidology</u> . 1986. (Medeiros-Neto <u>et al.</u> , eds.) Plenum Medical Books, New York. p 173-176. Entire Document.	57-60, 64-69
Y	J. Cell. Biol. vol. 106., April 1988. (Matzuk <u>et al.</u>) "The role of the asparagine-linked oligosaccharides of the subunit in the secretion and assembly of human chorionic gonadotropin". p 1049-1059. Entire Document.	57-60, 64-69
Y	J. Biol. Chem. vol. 264, No. 5, 15 February 1989 (Matzuk <u>et al.</u>), "Site specificity of the chorionic gonadotropin N-linked oligosaccharides in signal transduction". p 2409-2414. Note at least the abstract.	57-60, 64-69
Y	Molec. Endocrinol. vol. 2. 1988. (Matzuk <u>et al.</u>). "The glycoprotein -subunit is critical for secretion and stability of the human thyrotropin B-subunit". p 95-100. Entire document.	57-60, 64-69
Y	GB, 2091740 A (Katsuragi <u>et al.</u>) 04 August 1982. See at least page 1, second paragraph.	57-60, 64-69
Y	US, A, 4,780,451 (Donaldson) 25 October 1988. Entire document.	57-60, 64-69
Y	J. Clin. Endocrinol. Metabol. vol. 64, No. 2, 1987 (Jameson <u>et al.</u>)., "Gonadotropin and thyrotropin and B-subunit gene expression in normal and neoplastic tissues characterized using specific messenger ribonucleic acid hybridization probes". p 319-327. Note the abstract.	57-60, 64-69
Y	J. Biol. Chem. vol. 258. 19 October 1983. (Policastro <u>et al.</u>), "The B-subunit of human chorionic gonadotropin is encoded by multiple genes". p 11492-11499. See especially the abstract	57-60, 64-69
Y	Biochemistry. vol. 22, No. 13, 1983. (Keutmann <u>et al.</u>). "Chemically deglycosylated human chorionic gonadotropin subunits: Characterization and biological properties. p 3067-3072. See the abstract and the discussion.	57-60, 64-69

III. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of Document	Relevant to Claim No
Y	J. Biol. Chem. vol. 258, No. 1, 10 January 1983. (Kalyan <u>et al.</u>), "Role of carbohydrate in human chorionic gonadotropin". p 67-74. See the abstract, page 67, right column, and the discussion.	57-60, 64-69
Y	DNA, vol. 5, No. 5, 1986. (Maurer <u>et al.</u>), "Isolation and nucleotide sequence analysis of a cloned cDNA encoding the B-subunit of bovine follicle-stimulating hormone". p 363-369. See the abstract, discussion, and Figs. 2, 3.	57-60, 64-69
Y	J. Biol. Chem. vol. 246, No. 5, 10 March 1971. (Morell <u>et al.</u>), "The role of sialic acid in determining the survival of glycoproteins in circulation". p 1461-1467. See the abstract. See page 267, 268.	57-60, 64-49
Y	Science. vol. 229. 05 July 1985. (Sairam <u>et al.</u>), "A role for glycosylation of the subunit in transduction of biological signal in glycoprotein hormones. Entire Document.	57-60, 64-69
Y	J. Cell. Biol. vol. 104. May 1987. (Coriess <u>et al.</u>). "Gonadotropin beta subunits determine the rate of assembly and oligosaccharide processing of hormone dimer in transfected cells". p 1173-1181. See pages 1177-1181.	57-60, 64-69
Y,P	J. Biol. Chem. vol. 264, No. 9, 25 March 1989. (Keene <u>et al.</u>), "Expression of biologically active human follitropin in Chinese hamster ovary cells". p 4769-4775. Entire Document.	57-60, 64-69
Y,P	J. Biol. Chem. vol. 264, No. 29, 15 October 1989. (Bielinska <u>et al.</u>), "Site-specific processing of the N-Linked oligosaccharides of the human chorionic gonadotropin subunit". p 17113-17118. See at least the abstract.	57-60, 64-69
Y,P	Endocrinol. vol. 124, No. 4, 1989. (Saccuzzo <u>et al.</u>). "Identification and characterization of subpopulations of the free -subunit that vary in their ability to combine with chorionic gonadotropin-B". p 1613-1624. See entire document.	57-60, 64-69
Y	Arch. Biochem. Biophys. vol. 229, No 1, 1984. (Amr <u>et al.</u>). "Role of the carbohydrate moiety of human choriongonadotropin in its thyrotropic activity". p 170-176. See entire document.	57-60, 64-69
Y	Endocrinol. vol. 112, No. 2, 1983. (Chirm <u>et al.</u>) "Difference in sizes of human compared to murine -subunits of the glycoprotein hormones arises by a four-codon deletion or insertion". p 482-485. See entire document.	57-60, 64-69
Y	Biochim. Biophys. Acts. vol. 576. 1979. (Rathnam <u>et al.</u>). "Studies on modification of tryptophan, methionine, tyrosine, and arginine residues of human follicle-stimulating hormone and its subunits". p 81-87. Entire document.	57-60, 64-69

III. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of Document	Relevant to Claim No
Y	Adv. Exp. Med. Biol. vol. 205, 1986. (Boime et al.), "Structure and expression of human placental hormone genes". p 267-280. entire document, esp. pages 267-268.	57-60, 64-69
Y	US, A, 4,668,476 (Bridgham et al.) 26 May 1987 see entire document, esp. col. 1-	57-60, 64-69
Y	Ann. Rev. Biochem. vol. 57, 1988 (Kent) "Chemical synthesis of peptides and proteins". p. 957-989, See especially p. 984, 983, 985	57-60, 64-69

PCT/US90/01037

Attachment to: PCT/US90/01037

PCT/ISA/210
Part II, Fields Searched

Search Terms

FSH

Follicle Stimulating hormone

LH

Lutenizing hormone

CG

Chorionic gonatotropin

TSH

Thyroid stimulating hormone

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out¹, specifically:

3. ☐ Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:

See attachment

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

Claims: 57-60 and 64-69

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

Attachment to form PCT/ISA/210, Part VI

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

- I. Claims 1-11, drawn to a recombinant expression system, host cells, method for expressing a modified LH hormone beta subunit polypeptide and mutations thereof, and the polypeptide are for example, classified in Class 435, subclasses 69.4, 91, 320, and 252.3 and Class 536, subclass 27, and Class 530, subclasses 397 and 398.
- II. Claims 12-21, and 62, drawn to recombinant expression systems, host cells, method for expressing a modified LH hormone beta subunit the human glycoprotein and an unmodified alpha subunit polypeptide and mutations thereof, and the LH dimer are for example, classified in Class 435, subclasses 69.4, 91, 320, and 252.3 and Class 536, subclass 27, and Class 530, subclasses 397 and 398.
- III. Claims 22-24, drawn to a recombinant expression system and host cell for expressing a human alpha glycoprotein subunit and method to produce the human alpha subunit by culturing cells under conditions where the alpha subunit gene is expressed and recovering the human alpha subunit polypeptide are for example, classified in Class 435, subclasses 69.4, 91, 320, 252.3 and Class 536, subclass 27.
- IV. Claims 25-31 drawn to recombinant expression systems and host cells, a method to produce recombinant human alpha subunit and the beta subunit of FSH, LH, TSH, or CG, and the recombinant hormones per se, are for example, classified in Class 435, subclasses 69.4, 91, 320, and 252.3 and Class 536, subclass 27, and Class 530, subclasses 397 and 398.
- V. Claims 32-35, and 43, drawn to a mutein of the human glycoprotein alpha subunit lacking a glycosylation site a position 52 or 78 or both and/or lacks one or more amino acids 88-92, a recombinant expression systems and host cells, and method to produce recombinant human alpha subunit are for example classified in Class 435, subclasses 69.4, 91, 320, and 252.3 and Class 536, subclass 27, and Class 530, subclasses 397 and 398.
- VI. Claims 36-42, and 61, drawn to host cells, recombinant expression systems having a DNA encoding a human glycoprotein mutein and also having an expression system for a normal beta subunit of FSH, LH, TSH, or

Attachment to form PCT/ISA/210, Part VI

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

CG, and the peptide hormones per se, are for example classified in Class 435, subclasses 69.4, 91, 320, and 252.3 and Class 536, subclass 27, and Class 530, subclasses 397 and 398.

VII. Claims 44-48, drawn to an FSH extended at the C-terminus by the CTP sequence downstream of amino acid 111 of human chorionic gonadotropin beta, the host cells containing the expression system for the modified polypeptide and method of producing the C-terminal extended FSH are for example, classified in Class 435, subclasses 69.4, 69.7, 91, 320, and 252.3 and Class 536, subclass 27, and Class 530, subclasses 397 and 398.

VIII. Claims 49--56 and 63, drawn to recombinant host cells and expression systems having DNA encoding a chain extended FSH and a normal alpha subunit, the method of obtaining the hormone and the peptide hormone are for example, classified in Class 435, subclasses 69.4, 69.7, 91, 320, and 252.3 and Class 536, subclass 27, and Class 530, subclasses 397 and 398.

IX. Claims 57-60, and 64-69, drawn to pharmaceutical compositions of mixtures of FSH, LH, TSH, or CG, are for example, classified in Class 530, subclasses 397 and 398.

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